Microspheres for bone regeneration and localised delivery of enzymes

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To Vasco and Gonçalo,

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Abstract

Successful reconstruction of bone defects is a major concern in orthopaedic surgery and remains an active area of basic and clinical research. In recent years, increasing efforts have been devoted to the development of injectable materials that can be applied through minimally invasive surgery, providing less discomfort to patients, a faster recovery and lower costs. Their effectiveness can be improved through association with therapeutic agents, which may be locally released to accelerate the process of bone regeneration. Particle-based materials have already been used as injectable bone fillers. The size and shape of the particles dictate their spatial rearrangement within the cavity, playing a critical role in new bone formation, and it has been suggested that spherical-shaped particles with a narrow size distribution may present some advantages compared to other types of microparticles.

The main objective of this work was to develop microspheres with a uniform size, to be used as bone-defects fillers and as carriers for enzymes. As a specific application, the use of the system as an adjuvant therapeutic strategy to treat bone lesions associated with Gaucher disease (GD) was investigated, and microspheres were analysed as vehicles for the recombinant enzyme glucocerebrosidase (GCR), currently employed in its treatment.

As a starting point, alginate was used as a model-matrix for the entrapment of recombinant GCR. GCR-loaded microspheres with a uniform size were prepared by droplet extrusion and ionotropic gelation with Ca^{2+}. The enzyme retained full activity, and its stability at physiological pH was improved. GCR release profile was characterised by a high burst, followed by a stage of very slow release. The released enzyme was internalised by cultured GCR-deficient fibroblasts obtained from a GD patient, enhancing their residual enzymatic activity.

As a strategy to modulate GCR release-kinetics and at the same time improve cell adhesion to alginate microspheres, hydroxyapatite (HAp) or calcium titanium phosphate (CTP) were added to the polymeric matrix. Calcium phosphate-alginate microspheres were prepared, using the same technique. GCR was incorporated in the microspheres before gel formation in two different ways: pre-adsorbed onto the ceramic particles or dispersed in the polymeric/ceramic paste. The two strategies resulted in distinct release profiles, being the former characterised by a lower initial burst and a more gradual release.

Adhesion of osteoblastic-like cells to calcium phosphate-alginate microspheres of different compositions was analysed. The ceramic-to-polymer ratio strongly influenced the ability of cells to adhere and spread on the microspheres surface. Cells were only able to adopt a flattened morphology on microspheres with a high percentage of ceramic.

In view of these findings, microspheres composed only of calcium phosphate appeared to be suitable for the envisaged application. CTP and HAp microspheres were obtained by sintering calcium phosphate-alginate microspheres in order to burn-off the polymer and bond together the ceramic granules, while maintaining the spherical-shape of the particles. The microspheres were characterised using different techniques, and *in vitro* studies revealed that they can be used as supports for the culture of osteoblastic-like cells.

The possibility of coupling the enzyme GCR to CTP and HAp microspheres using adsorption as an immobilisation method was considered. In order to investigate some fundamental aspects of the interaction of recombinant GCR with solid surfaces, some mechanistic studies using self-assembled monolayers (SAMs) of different functionalities were used to investigate the effect of surface chemistry and solution pH on GCR adsorption behaviour. Depending on the conditions used for adsorption, the integrity of the enzyme could be preserved and in some cases its catalytic efficiency could even be improved.

Finally, some preliminary results indicate that calcium phosphate microspheres may be used to reversibly adsorb the enzyme while preserving its biological activity, and efficiently deliver it *in vitro* into GCR-deficient cells from GD type I patients. This suggests that physical adsorption of GCR to calcium phosphate microspheres may provide a simple and efficient method to couple the enzyme to the microspheres, and may enable its localised delivery to bone defects.

Resumo

A Reconstrução de defeitos ósseos é considerada um problema maior em ortopedia, e a investigação nesta área mantêm-se activa, a nível clínico e fundamental. Recentemente, têm-se vindo a empregar esforços no sentido de se desenvolverem materiais injectáveis que possam ser implantados de uma forma minimamente invasiva. Deste modo, é possível diminuir o desconforto para o paciente bem como os custos associados ao procedimento, e promover uma recuperação mais rápida. A eficácia destes materiais poderá ser melhorada se lhes forem associados agentes terapêuticos adequados, que uma vez libertados localmente no defeito ósseo irão acelerar a sua regeneração.

Materiais sob a forma de pequenas partículas têm sido usados como injectáveis. O tamanho e formato das micropartículas ditam o modo como estas se organizam espacialmente no interior da cavidade, influenciando o processo de formação de novo osso. Vários estudos sugerem que partículas esféricas com uma distribuição de tamanhos apertada apresentam vantagens relativamente a outro tipo de partículas.

O objectivo principal deste trabalho foi desenvolver micropartículas esféricas, de tamanho uniforme, para serem usadas como material de preenchimento de defeitos ósseos e como veículos de enzimas. Especificamente, estudou-se a sua aplicação como um método terapêutico adjuvante para o tratamento de lesões ósseas associadas à doença de Gaucher. Por esse motivo, as microesferas foram utilizadas na imobilização da enzima recombinante glucocerebrosidase (GCR), actualmente utilizada no tratamento desta doença.

Como ponto de partida, utilizou-se alginato como uma matriz modelo para a imobilização da enzima. Microesferas com um tamanho uniforme contendo a enzima foram preparadas utilizando a técnica de extrusão gota-a-gota seguida de gelificação na presença de Ca^{2+}. A enzima reteve a totalidade da sua actividade biológica e a sua estabilidade a pH fisiológico foi melhorada. O perfil de libertação da GCR caracterizou-se por uma etapa inicial de libertação rápida, seguida de uma etapa de difusão muito lenta. Foram realizados estudos *in vitro* utilizando fibroblastos de Gaucher com uma actividade da GCR endógena inexistente ou residual. A enzima libertada foi internalizada pelas células, resultando num aumento significativo da actividade enzimática intracelular.

Por forma a modelar a cinética de libertação da GCR e, simultaneamente, melhorar a adesão celular às microesferas de alginato, foram adicionados fosfatos de cálcio

(hidroxiapatite, HAp; ou fosfato misto de cálcio e titânio, CTP) ao polímero.

Microesferas de fosfato de cálcio/alginato foram preparadas usando a técnica anteriormente descrita. A enzima GCR foi incorporada nestas microesferas de dois modos: pré-adsorvida aos pós cerâmicos ou dispersa na pasta de polímero/cerâmico. As duas estratégias resultaram em perfis de libertação distintos, sendo o primeiro caracterizado por uma difusão mais gradual e uma diminuição da quantidade libertada na etapa inicial.

A adesão de osteoblastos a microesferas de fosfato de cálcio/alginato de diferentes composições foi analisada. A proporção cerâmico-polímero influenciou significativamente a percentagem de adesão e a morfologia das células. Só se observaram células esticadas e fusiformes em microesferas com elevadas percentagem de cerâmico.

Estes resultados sugeriram que microesferas compostas somente por fosfatos de cálcio poderiam ser adequadas para a aplicação pretendida. Microesferas de CTP e HAp foram obtidas por sinterização de microesferas de fosfatos de cálcio/alginato. Durante este processo o alginato é queimado e desaparece, ao mesmo tempo que os e grânulos de cerâmico se unem, mantendo-se a forma esférica das micropartículas. As microesferas foram caracterizadas por diferentes técnicas e estudos *in vitro* revelaram que as mesmas podem ser usadas como suporte de cultura de osteoblastos.

A possibilidade de se associar, por adsorção, a enzima GCR às microesferas foi tida em consideração. Alguns aspectos fundamentais da interacção da GCR com superfícies sólidas foram primeiro estudados usando superfícies modelo. Para isso utilizaram-se monocamadas auto estruturadas (SAMs) com diferentes grupos funcionais. Os efeitos da química da superfície e do pH da solução no processo de adsorção foram investigados. Dependendo das condições usadas, foi possível preservar a integridade da enzima e até melhorar a sua eficiência catalítica.

Ensaios preliminares mostraram que as microesferas de fosfatos de cálcio podem ser usadas para imobilizar a enzima GCR de um modo reversível, preservando a sua actividade biológica. A imobilização por adsorção constitui assim um método simples e eficiente de associar a enzima às microesferas e poderá permitir a sua libertação localizada em defeitos ósseos.

Résumé

La reconstruction des défauts osseux est considérée un problème majeur en orthopédie et jusqu'à présent est un domaine de recherches fondamentales et cliniques. Des efforts sont focalisés sur le développement de matériaux destinés à des applications cliniques par chirurgie mini invasive qui permettent à la fois une réduction des complications invalidantes pour le patient, une récupération assez rapide ainsi qu'une diminution des coûts. L'efficacité de ces matériaux peut être améliorée par une intégration d'agents thérapeutiques permettant le relarguage in situ de principe actifs et l'accélération du processus de régénération osseuse. Des biomatériaux à base de particules ont été déjà utilisés pour le comblement des défauts osseux. La taille et la forme de ces particules influencent leur distribution/disposition au sein du défaut osseux et par conséquent leur confère un rôle important dans le néoformation de l'os. Il a été suggéré que des particules en forme sphérique avec une distribution de taille réduite peut présenter des avantages en comparaison avec les autres types de microparticules.

Le principale objectif de ce travail était de mettre au point des microspheres de taille uniforme afin d'être utilisées comme matériaux de comblement des défauts osseux et de support pour des molécules tellques les enzymes. L'utilisation de ce système comme stratégie thérapeutique adjuvante pour le traitement des lésions osseuses associées à la maladie de Gaucher a été étudiée. Les microspheres ont été analysées comme support de l'enzyme glucocerebrosidase recombinante (GCR), actuellement utilisées dans les traitements cliniques de cette maladie.

Dans la première étape de ce travail, l'alginate a été utilisé comme modèle de matrice pour l'incorporation de GCR. Des microspheres de taille uniforme, chargées de GCR, ont été préparé utilisant la technique d'extrusion goutte à goutte suivie de gélification en présence de calcium. L'enzyme garde la totalité de son activité biologique et son stabilité au pH physiologique a été amélioré. Le profile de relarguage de GCR se caractérise par une étape initiale de libération rapide suivie par une étape de diffusion plus lente. Des études *in vitro* utilisant des fibroblastes de Gaucher avec une activité endogène de GCR inexistante ou résiduelle ont été réalisé. L'enzyme relarguée a été internalisée par les cellules, donnant lieu à une augmentation significative de l'activité enzymatique intracellulaire.

De manière à modeler la cinétique de relarguage de GCR et, simultanément, améliorer

l’adhésion cellulaire aux microspheres d’alginate, des phosphates de calcium (hydroxyapatite, HAp; ou phosphate mixte de calcium et de titanium, CTP) ont été additionné au polymère.

Les microspheres de phosphate de calcium/alginate ont été préparé utilisant des techniques antérieurement décrites. L’enzyme GCR a été incorporé à ces microsphères de deux manières: pre-adsorbée á la poudre de céramique ou dispersée dans la pâte de polymère/céramique. Les deux stratégies résultent en deux profiles de relarguage distincts; le premier caractérisé par une diffusion plus graduelle et une diminution de la quantité relarguée au cours de l’étape initiale.

L’adhésion des ostéoblastes aux microsphères de phosphates de calcium/alginate de différentes compositions a été analysée. La proportion céramique/polymère influence significativement le pourcentage d’adhésion et la morphologie des cellules. Sont observées les cellules allongées et fusiformes uniquement sur des microsphères avec un pourcentage élevé de céramique.

Ces résultas suggèrent que les microsphères composées uniquement de phosphate de calcium peuvent être adaptées à l’application clinique prétendue. Les microsphères de CTP et d’HAp ont été obtenues par sintérisation de microsphères de phosphate de calcium/alginate. Au cours de ce processus l’alginate est brûlée et ainsi disparaît. Au même temps les granules de céramiques s’unissent et les particules maintiennent une forme sphérique. Les microsphères ont été caractérisées par différentes techniques. Les études in vitro montrent qu’elles peuvent être utilisées comme support pour les cultures des ostéoblastes.

La possibilité d’associer, par adsorption, l’enzyme GCR aux microsphères a été prise en considération. Certains aspects fondamentaux d’interactions de GCR avec des superficies solides ont été premièrement étudiés utilisant des modèles de superficie. Dans ce but, des monocouches auto structurées (SAM) possédant différents groupes fonctionnels ont été utilisé. Les effets de la composition chimique de la superficie et du pH de la solution pendant le processus d’adsorption ont été étudiés.

Selon les conditions utilisées, il a été possible de préserver l’intégrité de l’enzyme et même améliorer son efficacité catalytique.

Des études préliminaires montrent que les microsphères de phosphates de calcium peuvent être utilisées pour immobiliser la GCR de façon réversible, préservant son activité

biologique. L’immobilisation par adsorption constitue une méthode simple et efficace permettant l’association de l’enzyme aux microsphères. Il est possible que cette méthode permettra le relarguage l’enzyme de manière localisée au niveau des défauts osseux.

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Chapter I

INTRODUCTION: MICROSPHERES FOR BONE REGENERATION AND LOCALISED DELIVERY OF ENZYMES

1. Microspheres as injectable materials for bone regeneration

1.1. General aspects

Several congenital anomalies, diseases and trauma may result in bone loss. In many situations, the damaged tissue fails to regenerate or heal spontaneously, creating the need to fill defects in the skeleton. The use of autografts to restore original function, although considered by many surgeons to be the best option, is limited by the reduced availability and the risks associated with harvesting.[1,2] On the other hand, allografts obtained from human donors, and xenografts obtained from other species exhibit limited regeneration potential, and can lead to immunorejection, and transmission of diseases.[2]

The field of regenerative medicine has emerged as an alternative strategy that combines engineering and life sciences principles, to create biologically functional substitutes aiming at restoring, maintaining or improving tissue function.[3,4]

Most strategies rely on the use of scaffold materials to provide a physical support for new tissue formation. The material can be seeded with cells prior to implantation, or implanted directly into the injured tissue and stimulate the host cells to promote local tissue repair.[5] Depending on the application, signalling molecules, such as growth factors, can be incorporated in the materials and improve their performance by stimulating the appropriate cells to migrate into the implant site, proliferate, and even differentiate along the desired lineage. In the ideal case, the material should degrade and resorbe, at a controlled rate, while being progressively replaced by histologically and functionally normal tissue.

Although the majority of scaffolds described in the literature consist of block tri-dimensional porous structures, in the past few years increasing efforts have been devoted to the development of injectable biomaterials as an alternative for bone augmentation or

replacement. The main advantage of using injectable materials is the possibility of filling bone-defects of different shapes and sizes using a minimally invasive surgery, which provides less discomfort to patients undergoing a variety of orthopaedic procedures, allows a faster recovery and has lower costs.[6]

Some of the desirable features of a biomaterial for bone regeneration applications include:[7-9]

- Be easily sterilisable to prevent infection, without losing key physicochemical properties;
- Be easily available ("off-the-shelf") and easy to handle by the surgeon;
- Be biocompatible, i.e., it must not elicit an unresolved inflammatory response nor be excessively immunogenic or cytotoxic;
- Have adequate initial mechanical properties to provide proper support in the early stages of healing, without compromising the load-bearing capabilities of the newly formed tissue;
- Promote the formation of a functionally normal tissue, identical to the original;
- Promote, or at least permit, the growth and maturation of blood vessels on the implant site, to guarantee an adequate supply of nutrients and oxygen to the newly formed tissue;
- Be biodegradable, i.e., it should degrade *in vivo* at a predictable and controlled rate, in concert to tissue growth, leading to biocompatible and ideally resorbable degradation products.

Additionally, an injectable material must present adequate rheological properties. These must be balanced, so that the material can be easily manipulated during surgery, but at the same time be adaptable to the irregular wound site, were it must remain for an adequate period of time. Some of the requirements that an injectable material must ideally satisfy to be used as a bone defects filler are listed below:[6,10]

- It must present an adequate fluidity to be extruded through the injection cannula without blocking it;
- It must have an adequate viscosity to allow the correct filling of the cavity;

- It must enable the regulation of the amount injected using a monitoring system, for example by being sufficiently radiopaque, so that the injection may be stopped the moment the defect is filled, without overflow;
- It must remain within the cavity, during an adequate period of time, withstanding blood flow and mechanical stress.

A variety of injectable materials, both ceramic- and/or polymer-based, have been recently described in the literature. Most of them consist of self-setting cements,[11,12] gels[13] and liquid precursors that solidify *in-situ* under physiological conditions or in response to an external stimulus.[14-16]

Micro- or nanoparticles suspended in autologous blood or other appropriate vehicle may also be used as injectable fillers for the regeneration of bone defects.[17-22] Upon implantation, the microspheres-vehicle system is expected to easily conform to the irregular implant site, providing a permissible matrix for both tissue and vascular ingrowth, as required for effective healing. The particles may, in addition, be seeded with autologous cells prior to implantation, functioning as cell-carriers, and/or be loaded with bioactive agents to stimulate the process of tissue regeneration. These different approaches will be discussed in the following sections.

1.2. Microspheres as bone defects fillers

Successful closure of bone defects remains a major concern in reconstructive surgery. A wide variety of particle-based fillers have been described in the literature, including irregular multifaceted granules and smooth spherical particles of various sizes, with or without pores, and with broad or uniform size distributions.[12,23-27] Depending on their characteristics, microparticles may find application in dental, periodontal, oral/maxillofacial and other orthopaedic surgical procedures.

While block-based osseous fillers may form sparse bone at their periphery and even less bone in their interior, an assembly of particles may allow bone regeneration throughout its volume cross-section. However, the size and shape of the particles dictate their spatial rearrangement within the implant site, playing a critical role in new bone formation.[28]

Although some empty space is necessary to allow tissue and blood vessels ingrowth, excessive interstitial space, resulting from the use of very large particles, may favour the development of fibrous tissue and compromise adequate healing.[29] In addition, large particles are more difficult to inject, leading to a more invasive implantation.
On the other hand, very small particles, as well as particles with broad size distributions, result in dense packing at the defect, which may impair tissue ingrowth and vascularization.[28] Depending on their size, very small particles may even be able to migrate from the implant site causing injury elsewhere.

The shape of the microparticles is equally important. Irregularly shaped particles have sharp or angular edges that are more susceptible to wear and may damage the surrounding tissue once implanted and/or induce strong inflammatory responses.[30]
The effect of particles size and shape on the degree of bone regeneration has already been studied by several authors.[28-31] For example, Mankani *et al.*[28] created a spectrum of roughly spherical particles of hydroxyapatite/tricalcium phosphate, with sizes in different ranges, from less than 44 µm to 1000-2000 µm. The particles were implanted along with human bone marrow cells in subcutaneous pockets in mice, and the type and extent of bone formation was estimated using different techniques. The best results were obtained when particles in the ranges of 100-250 µm and 250-500 µm were used. Particles were closely packed, with abundant bone and haematopoietic tissue occupying most of the interstitial space, and new bone fixing adjacent particles to each other. Largest particles in the ranges of 500-1000 µm and 1000-2000 µm were found to be widely separated by non-mineralized tissue. Bone tissue was sporadic and discrete and associated with individual particles. Transplants with the smaller particles, in the ranges of <100 µm, 44-62 µm and <44 µm, formed no bone.
Additionally, the same authors created flat-sided hydroxyapatite particles measuring 100-250 µm, which formed arrangements resembling closely packed stacks of plates and resulted in no bone formation and limited vascularisation.
In another study, Higashi *et al.*[31] showed that transplantation of hydroxyapatite and beta-tricalcium phosphate particles into hard tissue defects created in dogs resulted in superior bone formation around particles measuring 300 µm, while smaller particles with 40 µm were associated with less bone formation.

These examples highlight the relevance of particles size and shape on their ability to promote new bone formation, and suggest that spherical-shaped particles with a uniform and adequate size, which may be dependent on the application, present some advantages compared to other types of particulates described in the literature (Tab. 1).

Table 1. Possible disadvantages of some types of particulate materials.[28-30]

Type of microparticles	Disadvantages
Irregularly shaped particles	• Sharp edges that are more susceptible to wear and may damage the surrounding tissue once implanted • Inhomogeneous packing at the defect creating irregular voids • May induce a strong inflammatory response and delay tissue ingrowth[30]
Particles with a broad size distribution	• Less predictable properties during injection • Inhomogeneous and dense packing at the defect that may impair tissue ingrowth and vascularization[28] • Cells at different stages during *in vitro* colonization
Large particles	• More difficult to inject, more invasive implantation • Lower bone formation *in vivo* due to excessive interstitial space that may favour the development of fibrous tissue[28, 29]
Very small particles	• Dense packing at the defect that may impair tissue ingrowth and vascularization[28] • Migration of the particles from the implant site causing injury elsewhere

1.3. Microspheres as scaffolds for cell transplantation

For tissue repair strategies involving cell transplantation, isolated cells or small cell aggregates from the patient or a donor may be directly injected into the damaged tissue or combined with a scaffold before implantation.[4] When cells are injected as a suspension, their capacity to reform their respective tissue structure is limited since they lack a template to guide restructuring.[5, 32] A scaffold material may than be used a carrier matrix to deliver cells to specific sites in the body, to define a potential space in which new tissue may develop, to guide the process of tissue development, and even to elicit specific cell functions.[5, 33]

Although cells from different sources have already been employed for transplantation, therapies for bone repair generally involve the use of autologous bone marrow-derived stromal cells.[34] In most situations, these cells have to be expanded *in vitro* to allow the

generation of a large transplantable cell population from a small biopsy. When a scaffold material is used, cells may be seeded on the scaffold and directly implanted, or the cell-material construct may be further cultured to initiate tissue formation *in vitro* before implantation.[5, 33]

Depending on the application and type of material, the association of cells with the scaffold may be promoted in different ways. When microspheres are used, cells can be either entrapped or encapsulated within the particles (matrix or reservoir systems) or be seeded at their surface (microcarrier systems) (Fig. 1).

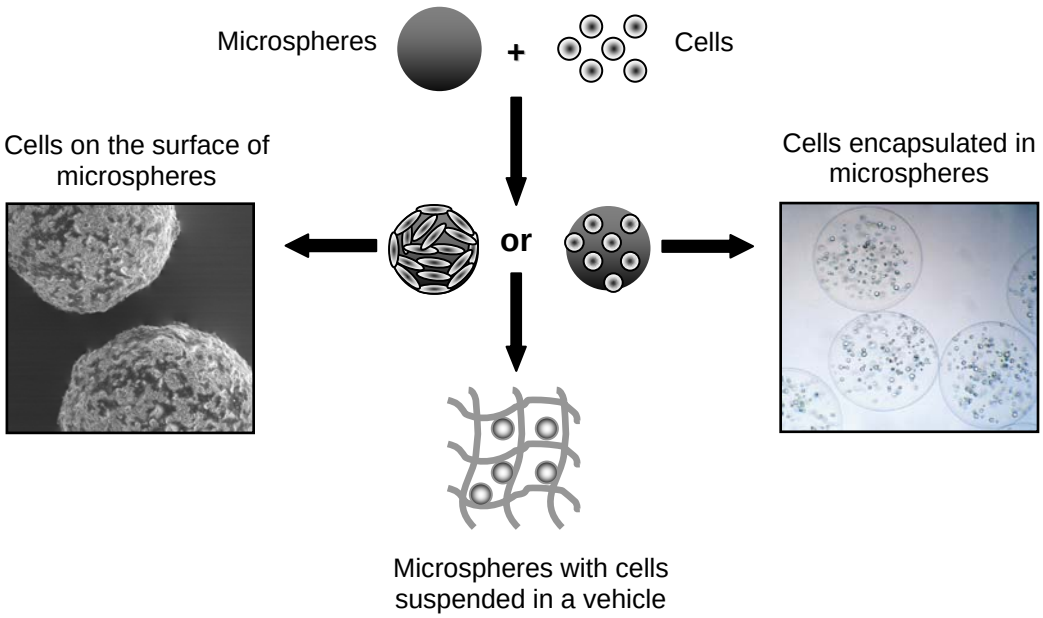

Figure 1. Schematic representation of microspheres loaded with cells: cells can be entrapped/encapsulated within the particles or be seeded at their surface.

Encapsulation and entrapment techniques are used to physically isolate cells from the outside environment, while maintaining normal cellular physiology within a desired permeability barrier.[35] For entrapment, cells are generally suspended in a gel or liquid precursor, which is subsequently crosslinked to form a spherical-shaped hydrogel network containing the dispersed cells. In microcapsules, cells become confined to a liquid core surrounded by a semi-permeable membrane.[35,36] In both cases, the system must allow oxygen and nutrients to diffuse in, and metabolites to diffuse out, in order to guarantee the maintenance of cells viability.[35]

Most of the examples described in the literature are intended for long-term immobilisation, and in most situations these matrix or reservoir systems are aimed at providing immunoisolation. A classical application is the immobilisation of insulin-secreting cells in polymeric microspheres, for the treatment of diabetes.[37]

Transplantation of cells within microspheres has also been used as a tool for tissue regeneration. In the field of orthopaedics, however, only a few examples have been described in the literature. For example, Spitzer *et al*. used microspheres composed of fibrin with or without α-tricalcium phosphate particles as a carrier for the entrapment and *in vitro* cultivation of periosteum-derived mesenchymal cells.[38] Cells were able to proliferate and differentiate along the osteoblastic lineage in both matrices, but exhibited an increased expression of osteocalcin, a commonly used bone phenotypic marker, when the ceramic particles were present.

Payne *et al*. described the entrapment of osteoblasts within fast-disintegrating gelatine microspheres, to be subsequently suspended in a poly(propylene fumarate) gel that solidifies *in situ*.[39-41] These microspheres were used to protect cells during the crosslinking process, which otherwise would affect cells viability. In this case, the microparticles are expected to degrade soon after implantation, liberating the cells.

Many cell types, however, perform poorly when suspended in a gel matrix losing their ability to proliferate or changing their phenotype. Consequently, some researchers have attempted to modify the scaffolds with bioactive moieties possessing inherent biological functionality in order to mimic, as close as possible, the natural extracellular matrix (ECM). One common example is the bulk functionalisation of hydrogel scaffolds with RGDs, which are short amino acid sequences that exist on integrin-binding proteins of the ECM and mediate the process of cell adhesion *in vivo*. In this sense, they are used to provide an improved environment for entrapped cells and also to promote the integration of the implanted cell-material construct after implantation. For example, Alsberg *et al*. showed that modification of alginate with RGD-containing peptides promotes osteoblast adhesion and spreading, whereas minimal cell adhesion is observed on unmodified hydrogels.[42] Furthermore, the authors demonstrated that transplantation of primary rat calvarial osteoblasts within alginate beads revealed statistically significant increases of *in vivo* bone formation with RGD-modified alginate compared with unmodified alginate. Burdick JA *et al*. promoted the photoencapsulation of osteoblasts in injectable poly(ethylene

glycol) (PEG) hydrogels, and demonstrated that entrapped osteoblasts were able to form a mineralized matrix, which was enhanced in gels grafted with RGD adhesive peptides.[43]

Anchorage-dependent cells may also be seeded on the surface of microspheres for subsequent transplantation. In this case, once implanted, cells may freely migrate and participate more actively in the process of tissue regeneration. The concept of culturing cells as monolayers on the surface of small spheres, commonly referred as microcarriers, was first conceived by Van Wezel[44] and different types of microcarriers are currently available in the market. The large surface area-to-volume ratio provided by microcarriers allows easy propagation and high cell yields, while requiring much less culture medium and space than traditional monolayer culture techniques. In cell culture technology, microparticles have been used as an alternative culture-substrate for the *in vitro* growth of anchorage-dependent cells, for large-scale cell expansion and/or production of numerous cell products including vaccines, enzymes, antibodies, etc. More recently, attention has been drawn to the use of microparticles as injectable cell-carriers for tissue regeneration, including bone.[17, 28, 45-49]

For example, Hsu *et al*. prepared microspheres comprised of particulate hydroxyapatite dispersed in a fibrous collagen matrix and used them as supports for the *in vitro* growth of osteoblasts isolated from rat calvaria.[17] The cells proliferated along 7 days in culture and expressed alkaline phosphatase activity. Further studies demonstrated that osteoblasts cultured on these microspheres were also able to form a mineralized matrix.[49]

1.4. Microspheres as vehicles for bioactive molecules

Bone repair is a complex cascade of biological events regulated by specific cells, the extracellular matrix and distinct growth factors, which act as signalling molecules and modulate cell function.

The biochemical stimulation of tissue regeneration via the local delivery of exogenous growth factors is currently one of the strategies used in bone repair therapies.[50] By selecting the appropriate growth factors, the relevant type of cells may be induced to migrate into the implant site, proliferate and finally differentiate along the desired lineage.

Some of the most important growth factors with potential for bone regeneration include the various bone morphogenetic proteins (BMPs), the transforming growth factor beta (TGF-β), the insulin-like growth factor (IGF), the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF).[51] Their biological and clinical roles in skeletal growth and bone formation have been extensively investigated.[52, 53]

However, the fragile nature of these proteinaceous agents, namely their short biological half-life and lack of long term stability, has limited their use for therapeutic purposes and has motivated the development of new delivery strategies based on the use of protective carriers.[54, 55] The utilization of vehicles may also enable the simultaneous administration of a combination of growth factor with synergistic effects, and coordinate their actions by providing a controlled release kinetics in a temporal and tightly regulated sequence.[54, 56]

Numerous examples of the use of microparticles as growth factors carriers for use in bone repair may be found in the literature.[57-59] Depending on the specificity of the illness, other bioactive agents such as antibiotics and other pharmaceutical agents may also be incorporated in bone-defect fillers to improve their performance and enhance tissue regeneration.

In the next section, an overview of the present technology used in the preparation of protein delivery systems, including a description of the most common protein-carrier coupling techniques, is provided. Protein stability in controlled release formulations and release mechanisms are also discussed.

2. Protein delivery systems

2.1. General aspects

During the past few years, remarkable advances in the area of biotechnology have allowed several genetically engineered protein drugs to be produced, which are now commonly used to treat numerous diseases.[60] Despite their therapeutic potential, their clinical application is in many situations hampered by their unstable nature, high susceptibility to degradation and short *in vivo* half-life. For this reason, the effectiveness of these protein agents is in general highly dependent of a frequent-administration regimen, which compromises the patient's quality of life, and often results in very expensive treatments.
The development of new delivery routes, based on the use of protein vehicles, may suppress some of these shortcomings and improve the performance of protein pharmaceuticals, by providing an optimized therapy and better patient compliance. Some of the potential advantages that protein delivery systems may offer are listed below:[61-64]

- Continuous or pulsatile protein release over extended periods of time;
- Protection of protein drugs from rapid *in vivo* degradation and clearance, prolonging their half-lives and consequently their efficiency;
- Avoidance of irregular plasma levels, with peaks and valleys, associated with conventional injections;
- Localised delivery of proteins to targeted sites, body compartments or cell types, reducing their overall systemic exposure;
- Self-regulation, providing periodic protein release when physiologically desirable or protein release triggered by an external stimulus.

In the future, gene therapy may be able to replace protein therapeutics, but currently there are still considerable technical obstacles. For this reason, in the near future protein drugs are expected to retain or increase their importance, and with improvements in formulation and administration routes they may never become obsolete.[63]

2.2. Protein-carrier coupling techniques

Most of the techniques used in the preparation of protein delivery systems rely on the association of proteins with soluble or solid carriers. There are several protein-carrier coupling strategies, which have been applied particularly within the field of enzyme immobilisation[65] and, more recently, for controlled release of protein pharmaceuticals.[61]
Some of the most commonly used protein coupling techniques are described below. The first two approaches are based on the establishment of physical or chemical bonds between protein molecules and a solid surface. The other approaches involve the physical entrapment of proteins within polymer lattices or microcapsules, and the formation of protein-polymer soluble conjugates. Finally, the last category consists on the association of proteins with biological structures such as liposomes and cells.[66]

2.2.1. Physical and chemical attachment to a surface

Non-specific physical adsorption of a protein from a solution on a solid surface is probably the simplest and longest known method of immobilisation. It requires little more than bringing the solid phase into contact with a protein solution for a short period of time. So, the ability to load the protein in a simple and cost-effective process, without using solvents or any polymerisation steps, is one of the greatest advantages of this method.[65,67]
Proteins have different surface domains, with hydrophobic/hydrophilic and negatively/positively charged character, and as a consequence they are highly surface active. The most relevant phenomena involved in the process of protein adsorption are electrostatic and hydrophobic interactions, and also the intrinsic structural stability of the protein.[68,69] The rate and extent of adsorption will be determined by the properties of the protein and its concentration in solution, by the characteristics of the adsorption matrix, and by the nature of the solvent and other properties of the medium, including pH, ionic strength and temperature.[68] Proteins usually have higher affinity for moderately hydrophobic surfaces than for hydrophilic ones.[68]
Upon adsorption, proteins generally undergo structural rearrangements dependent on the type and strength of the interactions established with the matrix.[70-72] Conformational alterations may result on protein denaturation and/or loss of functional activity[73, 74] or, in opposition, provide enhanced stability and/or biological functionality to the protein.[71, 75-79]

Physical adsorption methods have been widely used in the development of microparticulate carriers for the localised delivery of vaccines to the site of action,[80-83] and for the delivery of proteinaceous agents in tissue regeneration applications.[18, 84-86]

Although adsorption techniques are characterised by their practical simplicity, they lack the potential specificity of covalent techniques. Chemical immobilisation processes may be designed to induce protein molecules to be specifically bound in a favourable orientation, while allowing the retention of functional activity.[87] Proteins possess different reactive groups available for chemical bonding, namely amines, carboxyl groups and thiols, as well as carbohydrate moieties in the case of glycoproteins.[65] Different supports, with functional groups suitable for covalent coupling or susceptible to activation, have already been used.[65]
A chemically immobilised protein may also be attached to the surface via a spacer group, which can provide greater steric freedom and thus greater biological functionality to the protein.[67]
Although protein immobilisation through covalent attachment may result in loss of activity, chemical bonds are generally very stable and may confer higher thermal and storage stability to the immobilised protein by preventing structural rearrangement during use.[65]

2.2.2. Entrapment and encapsulation within polymeric matrices

Entrapment or encapsulation within polymeric matrices, are probably among the techniques most widely used in the designing of proteins delivery systems. Different vehicles, with a variety of geometries and configurations, have been fabricated from diverse types of natural and synthetic polymers.[56,61,88-90] Micro- and nanoparticles are among the most popular forms since they can be applied through minimally invasive surgery.
The term “entrapment” is commonly used for monolithic systems in which the protein becomes dispersed throughout the matrix, while the term “encapsulation” is used for reservoir systems in which the protein becomes confined to a cavity surrounded by a semi-permeable membrane that regulates the release rate.
The protein to be immobilised can be loaded into the hydrogel matrices after or before polymerisation.[89] In the former case, loading is carried out by soaking a dried matrix in a

solution containing the protein. Usually, this procedure results in a rather heterogeneous distribution of the protein within the matrix, with a significant portion being localised at the surface. Moreover, loading of very high molecular weight proteins may be difficult, if their size prevent them to readily diffuse into the structure. Although this method may result in minor losses of protein integrity, the loading efficiencies that can be obtained are generally low.[89]

In the other case, when proteins are present during the polymerisation step, their integrity may be compromised if the conditions used are too harsh. However, many polymers, namely those which are water-soluble, are able to form gels under very mild conditions, allowing proteins to be entrapped while retaining full activity.[89]

2.2.3. Conjugation with soluble polymeric moieties

The conjugation of proteins with other species, such as different proteins and water-soluble polymers provides a valuable means of altering the biological behaviour of the protein of interest, and has been envisioned for a variety of purposes.[61, 91, 92] Among these are the ability to increase the hydrodynamic radius of the protein, which results in a decreased rate of renal clearance and a prolonged circulation half-life. Additionally, protein-polymer conjugates may exhibit increased solubility, lower immunogenicity and lower susceptibility to proteolysis than the isolated proteins.[61,93] One of the polymers most widely used in the formation of protein-polymer conjugates is poly(ethylene glycol (PEG).[94, 95] Conjugation with peptides and carbohydrate moieties has been used to assist in protein targeting to specific cell types.[96, 97]

2.2.4. Association with biological structures

Biological structures such as liposomes and cells also constitute potential carriers for the *in vivo* delivery of protein agents.

Liposomes were characterised for the first time by Bangham *et al.*[98] They were initially developed as models of biological membranes, and only after applied as drug delivery systems. Basically, liposomes are spherical lipid vesicles with one or more concentric lamellae, which are formed in aqueous solution on the dispersion of certain bilayer-forming molecules.[99] The hydrophobic part of the amphiphiles forms the hydrophobic

interior of the bilayers and the hydrophilic part, the polar head group, is in contact with the aqueous phase. The interior of the liposomes is an aqueous core with a chemical composition similar to the one of the solution used to prepare them. The chemical nature of the amphiphilic molecules and the method used to prepare the vesicles are selected taking into consideration the type of protein and the final application. Protein-containing lipid vesicles have been extensively applied in the biomedical field, particularly for enzyme-replacement therapies and delivery of vaccines.[99, 100]

Some cell types, in particular erythrocytes, have also been used as protein carriers.[101,102] Because of their biocompatible nature, these cells elicit little or none immune response. Additionally, they are readily available in large quantities and allow high entrapment efficiencies.[101,102] Since the physiological turnover of erythrocytes involves their destruction primarily by macrophages of the spleen and liver, they are commonly used to selectively target these organs.[101,102]

2.3. Protein stability in controlled release formulations

Protein stability is probably one of the most important obstacles for the successful formulation of protein-delivery systems.

Unlike other therapeutic agents, proteins are high molecular weight compounds with a defined structure, which is responsible for their unique biological functions. For this reason, proteins are very susceptible macromolecules that may easily become reversible or irreversible inactive by several mechanisms, such as unfolding, chemical alteration, self-aggregation, etc.[103-105]

During preparation, and throughout the lifetime of the delivery system, proteins may become exposed to potentially harmful conditions. Therefore, the degradation pathways and their impact on protein stability must be systematically analysed. Some common sources of protein inactivation are: interactions between the protein and the matrix, exposure of the protein to the conditions selected for the immobilisation process (type of solvents, temperature, pH, shear forces, etc.), and exposure of the protein to an altered environment associated with the degradation of the matrix (degradation products, pH, loss of excipients, etc).[61, 103, 104] These interactions will ultimately influence the release rate and the overall condition of the released protein.

Two major stability problems, often encountered after implantation of the delivery system and during the release period, are the formation of insoluble protein-protein aggregates, usually in entrapment systems, and the irreversible adsorption of the protein to the carrier.[106-108] While the formation of aggregates should be avoided as these are often therapeutically inactive and may be immunogenic, protein adsorption to the matrix should be reduced as much as possible, since a fraction of the valuable protein will be wasted. Fortunately, different strategies have been proposed to overcome these problems, namely through the control of formulation parameters and the use of protective excipients.[108-111]

2.4. Release mechanisms

Depending on the application of the delivery system, the objective may be either to a have a transient or a long-term association of the protein to the carrier. Release of proteins from their vehicles will be, however, highly dependent on the coupling method initially used. For example, physical adsorption bonds are generally reversible, at least partially, following ionic and pH changes and especially in the presence of other proteins.[112] Huber *et al*. have developed a sophisticated device with the ability to adsorb proteins from solution, hold them with negligible denaturation, and release them on command.[113] In this case, surface properties are modulated by an active polymer which can be thermally switched between a more hydrophobic protein-adsorbing state to an antifouling hydrophilic state that promotes protein release.

Covalent coupling of protein with soluble or non-soluble carriers, although generally irreversible in nature, may also be used for delivery purposes if biodegradable carriers or labile bonds are selected.[67]

Protein release from matrix or reservoir systems can be governed by several mechanisms that can be generally classified as: (a) pure drug diffusion through a rate-controlling membrane or matrix (diffusion controlled), (b) degradation of the matrix (degradation controlled) or (c) counter-current diffusion of aqueous medium into the matrix (swelling controlled). This are however simplified models, and release profiles are often more complex resulting from a combination of mechanisms.[61,89]

Some of the most commonly observed protein release profiles are illustrated in Fig. 2.[61] The zero-order profile is characterised by a constant release rate over time. The first-order

profile, typical of a diffusion-controlled system, is characterised by a release rate that decreases with time. The biphasic release profile, which often occurs in degradable matrices, is characterised by two distinct release phases: the first one is characterised by protein diffusion from the surface and through the porous network, until a plateau is reached; and the second one is observed posteriorly, when the matrix begins to degrade liberating the remaining drug. Finally, there is the burst profile, which may also precede the other depicted release profiles and can generally be attributed to surface localised or non-incorporated proteins.

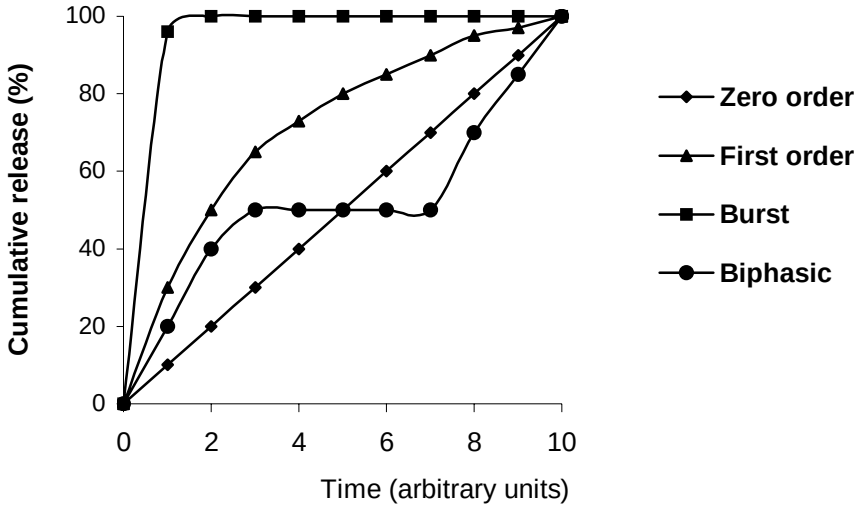

Figure 2. Different time-dependent release profiles commonly observed in protein delivery systems.[61]

Although major emphasis has been focused on the development of zero-order release systems, this profile is not always necessary and sometimes is not desirable. For example, a constant release over time may not match drug absorption by the body, which often decreases over-time.[89] Also, for certain therapies that require fluctuating protein levels, as for example the delivery of insulin for patients with diabetes, the amount of drug released should be affected according to physiological needs. Some polymers, generally designated as "smart" or "stimulus responsive" can respond to environmental or external stimulus that trigger drug release.[14,88,89,114] In self-regulated systems the release rate is controlled by feedback information, without any external intervention. Several approaches, such as pH-sensitive polymers, enzyme-substrate reactions, pH-sensitive drug solubility, competitive

binding and metal concentration-dependent hydrolysis, may be used as rate-control mechanisms.[114] In externally controlled systems, release may be modulated by external stimulus of different sources, such as: magnetic, ultrasonic, thermal, and electric.[114]

3. Gaucher disease

3.1. General aspects

Gaucher disease (GD) is an autosomal recessive disorder, characterised by the decreased catalytic activity and/or stability of the lysosomal enzyme glucocerebrosidase.[115-118] This defect leads to an excessive intracellular accumulation of glucosylceramide, a constituent of biological membranes and its major natural substrate.[115-118] Although glucocerebrosidase is present in almost all cell types, the accumulation of glucosylceramide is characteristically observed in macrophages, as a result of their role in the turnover of blood cells and consequently on the degradation of membrane glycolipids.
The hallmark of GD is the occurrence of lipid engorged cells, designated Gaucher cells (Fig. 3a), in several organs (Fig. 3b), underling the most common clinical manifestations of the disease, namely organomegaly, haematological abnormalities and skeletal deterioration.[115-118]

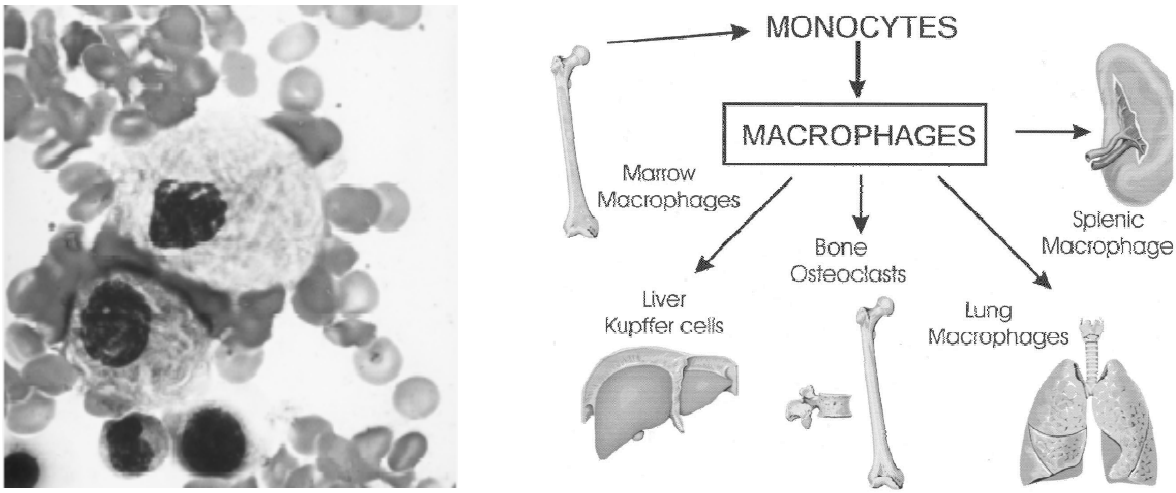

Figure 3. a) Gaucher cells in bone marrow aspirate[118] b) targeted organs in GD.[117]

Classically, three main variants of GD are considered on the basis of the presence and degree of neurological involvement. Type 1, the mildest and most common form, is characterised by the absence of neurological involvement and the onset of clinical manifestations may occur from childhood to adulthood. Type 2, is a rare variant causing

severe early-onset neurological manifestations usually fatal within 2 years of age, and type 3 is a subacute juvenile-onset slowly progressing neurodegenerative form.
The phenotypes and genotypes associated with GD are markedly heterogeneous, and there is a wide variability in the pattern and severity of disease involvement between and within each subtype. The most frequent mutation (N370S) is only present in type I GD patients.

GD is the most prevalent and best studied lysosomal storage disease. The subtype I occurs with a frequency of 1 in 40000-60000 in the general population, and in 500-1000 among Ashkenazi Jews.[119]
The birth prevalence of GD in Portugal is 1 in 73000.[120,121] Among the 84 diagnosed GD patients, 21 are homozygots for the N370S mutation.[120,121] However, according to the gene frequency determined in the general Portuguese population,[120] about 200 homozygots for the N370S mutation can be predicted. These findings suggest that the great majority of homozygotes for this mutation present a mild and/or non classical clinical phenotype, remaining undiagnosed.[120, 121]

3.2. Glucocerebrosidase

Glucocerebrosidase (β-glucosidase, *N*-acyl-sphingosyl-l-β-D-glucoside glucohydrolidase, EC 3.2.1.45) is an acid hydrolase present in lysosomes of all nucleated cells of mammals. It is responsible for catalysing the conversion of glucosylceramide (*N*-acylsphingosyl-l-O-β-D-glucoside), its major natural substrate, to ceramide and glucose, through hydrolysis of the O-β-D-glucosidic linkage (Fig. 4).

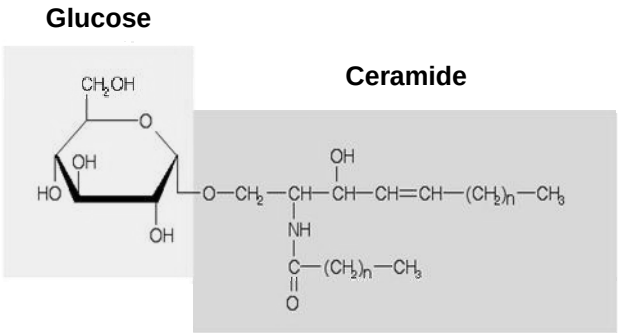

Figure 4. Structure of glucosylceramide (*N*-acylsphingosyl-l-O-β-D-glucoside).

In its natural physiological state, GCR is tightly associated to the inner membrane of lysosomes with substantial hydrophobic interaction, but no evidence of a transmembrane domain has been found.[122] The mature unglycosylated human enzyme has 497 amino acids (Tab. 2) and has five predicted N-glycosylation sites, being the first four normally occupied.[123] Studies with cultured human skin fibroblasts[124] showed that in those cells glucocerebrosidase is synthesised as a 62.5 kDa precursor with high-mannose-type oligosaccharide chains and an apparent native isoelectric point of 6.0-7.0. Subsequent processing of the oligosaccharide moieties to sialylated complex-type structures results in formation of 65-68 kDa forms of the enzyme with apparent native isoelectric points of 4.3-5.0. These forms are transported to lysosomes via unknown mechanisms and subsequently modified by the sequential action of lysosomal exoglycosidases, finally resulting in a 59 kDa form with an isoelectric point near neutrality. The lysosomal enzyme may exist in different aggregation states, whether as a monomeric form or as an active larger complex containing negatively charged phospholipids and activator proteins (saposine C).[125-127] Presumably, these compounds facilitate the interaction of the enzyme with the substrate by inducing a structural change that conforms it into a more active state (Fig. 5).[125,126]

Table 2. Amino acid sequence of human glucocerebrosidase.[128]

	1	11	21	31	41	51	
	|	|	|	|	|	|	
1	ARPCIPKSFG	YSSVVCVCNA	TYCDSFDPPT	FPALGTFSRY	ESTRSGRRME	LSMGPIQANH	60
61	TGTGLLLTLQ	PEQKFQKVKG	FGGAMTDAAA	LNILALSPPA	QNLLLKSYFS	EEGIGYNIIR	120
121	VPMASCDFSI	RTYTYADTPD	DFQLHNFSLP	EEDTKLKIPL	IHRALQLAQR	PVSLLASPWT	180
181	SPTWLKTNGA	VNGKGSLKGQ	PGDIYHQTWA	RYFVKFLDAY	AEHKLQFWAV	TAENEPSAGL	240
241	LSGYPFQCLG	FTPEHQRDFI	ARDLGPTLAN	STHHNVRLLM	LDDQRLLLPH	WAKVVLTDPE	300
301	AAKYVHGIAV	HWYLDFLAPA	KATLGETHRL	FPNTMLFASE	ACVGSKFWEQ	SVRLGSWDRG	360
361	MQYSHSIITN	LLYHVVGWTD	WNLALNPEGG	PNWVRNFVDS	PIIVSITKDT	FYKQPMFYHL	420
421	GHFSKFIPEG	SQRVGLVASQ	KNDLDVALM	HPDGSAVVVV	LNRSSKDVPL	TIKDPAVGFL	480
481	ETISPGYSIH	TYLWHRQ					

Abbreviations: A-Alanine, C-Cysteine, D-Aspartic acid, E-Glutamic acid, F-Phenylalaline, G-Glycine, H-Histidine, I-Isoleucine, K-Lysine, L-Leucine, M-Methionine, N-Asparigine, P-Proline, Q-Glutamine. R-Arginine, S-Serine, T-Threonine, V-Valine, W-Tryptophane, Y-Tyrosine.

Purification of GCR requires detergents for complete extraction. The lipid-depleted purified enzyme is essentially inactive *in vitro*, and in order to reconstitute its catalytic activity, the presence of exogenous activators such as detergents, bile salts or negatively charged phospholipids becomes essential.[126,129, 130] It exhibits an acidic pH optimum, around 5.5, under standard assay conditions, which is consistent to its lysosomal location.

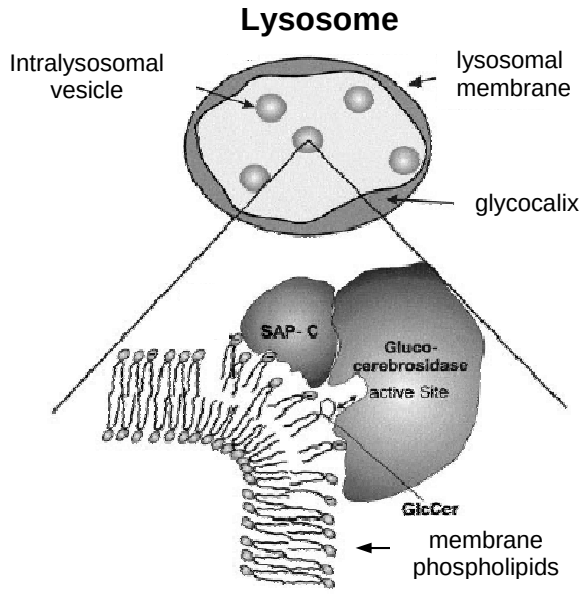

Figure 5. A proposed model for the degradation of membrane-bound glucosylceramide (GlcCer) by glucocerebrosidase and the activator protein saposin C (SAP-C).[126]

3.3. Therapeutic approaches for the management of GD

Different mutations on the gene that encodes for GCR give rise to the expression of mutant proteins with defective catalytic properties and/or stability, which ultimately result in the manifestation of several debilitating symptoms.[131] Besides palliative care, various treatments have been tried for the management of patients with GD. Some are still in use, and others have been discarded because of lack of effect or severe side-effects. The two most relevant currently available therapies are discussed in the next sections.

3.3.1. Currently available therapies: ERT and SRT

Enzyme replacement therapy (ERT): After the enzymatic defect has been demonstrated in GD,[132] consideration was given to the development of therapy for patients with this disorder, to prevent and/or correct the ongoing formation of lipid loaded Gaucher cells. The intravenous administration of exogenous GCR was proposed, and its rationale was based on the expected entry of the enzyme, through endocytosis, into the diseased cells and subsequent action *in situ*.[132] However, unmodified glucocerebrosidase derived from natural sources was of limited therapeutic value, due to its apparent inability to reach and/or act in the target intracellular organelles, or its wastage in non-targeted areas, and clinical trials were disappointing.[133,134]

With the discovery that macrophages express a receptor specific for α-mannosyl terminated oligosaccharides,[135, 136] attempts were made to modify the oligosaccharide chains of purified GCR in order to expose mannose residues, which could allow the targeting and intracellular trafficking of intravenously administered exogenous enzyme.[137] Improved delivery of human placental GCR to phagocytic cells was effectively achieved after treating the enzyme with neuraminidase, β-galatosidase and β-N-acetylhexosaminidase.[137]

These findings stimulated the commercial development and clinical use of an industrial formulation of mannose-terminated GCR derived from human placenta (Ceredase®), and the advent of enzyme replacement therapy in the early 1990s revolutionized the management of patients with GD.[138] More recently, an analogue of the human enzyme was produced by recombinant DNA technology, using Chinese hamster ovary (CHO) cell cultures.[128] Although both enzymes show a therapeutic similarity,[139, 140] the recombinant product (Cerezyme®) presents the advantage of being more easily obtained, as it is theoretically unlimited in supply, and of being free of potential pathogenic agents. For this reason, it gradually replaced the placenta-derived enzyme in the treatment of GD.[122]

After a decade of experience, the beneficial effects of ERT are clear, as it is clinically effective in halting the progression of GD and in reversing many of its debilitating manifestations. Several studies have been published and it has been well documented that ERT reduces organomegaly, improves haematological and biochemical indices, decreases bone related pain and induces compensatory growth in children.[118,122,141]

Substrate reduction therapy (SRT): A novel oral treatment based on the inhibition of substrate biosynthesis has been recently suggested and is currently on clinical trials.[142, 143] It involves the use of the iminosugar N-butyldeoxynojirimycin (NB-DNJ, Zavesca®), which is an inhibitor of glucosyltransferase, the enzyme that initiates the glycosphingolipid biosynthetic pathway and catalyzes the formation of glucocerebroside.[142, 143] NB-DNJ lowers the synthesis of glycosphingolipids, and consequently their accumulation, to rates at which the residual enzymatic activity, when existent, can catabolize stored and incoming substrate. Since this strategy is based on the use of a small molecule that is able to transverse the blood-brain barrier, it may be effective in the treatment of patients with neurological involvement.

3.3.2. Suggestions for improved or complementary therapies

Despite the unquestionable benefits of ERT and the recent availability of SRT, several issues remain unresolved concerning both treatments, consequently limiting their effectiveness.[144] Among these, are the dependence of ERT on a life-long frequent-administration regimen, poor delivery of exogenous enzyme to bones and lungs, and its inability to cross the blood-brain barrier.[144] In what concerns SRT, several adverse secondary effects have been described, probably related with the reduction on the level of several glycosphingolipids, and for that reason its use has been approved only for mild-to-moderate types of GD in cases where ERT is not suitable.[119,143] Moreover, both therapies are extremely expensive and therefore unavailable to patients in poor countries.
Positive results on a radically different strategy designated by enzyme enhancement therapy (EEC), which may address some of the problems of ERT, have recently been reported.[145,146] EEC is based on the use of small molecules as "pharmacological chaperones" to rescue misfolded and/or unstable mutant enzymes that have residual function. These compounds target the most common GD mutation (N370S). A therapy based on these chaperones has the potential to be much more convenient and less costly than ERT, because small molecules could be taken orally and would be cheaper to mass-produce than whole enzymes. In addition, small molecules can be found that cross the blood-brain barrier, and perhaps address the neurological complications of GD, if they are caused by mutants that respond to chaperone therapy.

ERT may also be improved by one of the following techniques, involving the production of less costly enzyme analogues and development of enzyme activators and/or stabilizers:

Transgenic analogues of GCR: The high cost of the recombinant enzyme currently used in ERT, considered as one of the world's most expensive drug,[147] seriously restricts its availability, especially to patients in poor countries. A new source of GCR, involving its expression in plant-based systems, as already been proposed as an economically more viable alternative.[147] Human GCR was engineered as a secreted protein in transgenic tobacco plant, and massive amounts of purified enzyme could be obtained.[147] Apart from economic advantages, the production of protein pharmaceuticals using transgenic plants is

easier to control and products are unlikely to be contaminated by animal pathogens, microbial toxins or oncogenic sequences.[148] However, transgenic GCR is not yet available in the market.

Modifications of GCR: Efficacy of GCR therapeutics could presumably be achieved by improving at least one of the following properties: 1) prolonged functional circulation half-life, 2) enhanced uptake by target cells, 3) improved intracellular trafficking to lysosomes and 4) prolonged intracellular functional half-life. This way, lower enzyme doses and/or less frequent administrations could lead to equal or better treatment efficacy in comparison to that obtained with conventional GCR therapeutics. Two strategies that have been proposed to achieve this are the modification of the enzyme through introduction of additional and/or specific glycosylation sites or its conjugation with non-antigenic polymeric moieties such as PEG.[149,150]

Carriers for GCR: The development of adequate carriers for the *in vivo* delivery of GCR could also improve its therapeutic potential. Some attempts to develop vehicles for this enzyme were made in the past, prior to the discovery that macrophages express specific receptors for endocytosis of mannose-terminal glycoproteins. Entrapment of GCR into different vehicles was then envisaged, to exploit the inherent phagocytic ability of target cells that would account for the internalisation of exogenous enzyme.[151-154]

Encapsulation of placental GCR in erytrocytes, coated with immunoglobulin to enhance specific uptake by macrophages, proved to be an effective strategy to target the enzyme to the relevant cells, but its clinical applicability was limited by the difficulties in purifying sufficient amounts of enzyme, and by the low entrapment efficiencies achieved.[151,152]

Gregoriadis *et al*. performed clinical trials with liposome-entrapped placental GCR on a patient with type I GD, but no significant improvements in the clinical condition of the patient were observed after a long-term treatment.[154] With the advent of ERT, and since clinical results obtained with liposome-entrapped placental GCR were disappointing, this approach was abandoned.

More recently, Korablyov *et al*. prepared liposome-entrapped recombinant GCR with high entrapment efficiencies, and suggested that it would be worthwhile to re-examine the use of this type of carriers, by taking advantage of the availability of pure and efficacious

industrial enzyme formulations and of the greatly improved understanding of liposome technology and biofate.[155] However, these studies were not further developed.

3.4. Bone involvement in GD

Skeletal lesions of GD are among its most debilitating consequences. Nearly all affected patients, approximately 80%, have some degree of bone involvement, and more than half may suffer from serious complications, including pathological fractures.[156-158] The skeletal aspects of GD have a much greater impact on patient's quality of life than the haematological and visceral abnormalities, by interfering with the activities of daily life and restricting mobility.[158,159]

The degree and type of bone lesions are highly variable. Bone disease can be asymptomatic even when severe, or be accompanied by acute or chronic pain.[157] A five-stage classification of the skeletal alterations normally observed in GD has been proposed by Herman G *et al.*, and is presented in Tab. 3.[160]

Table 3. Staging of skeletal alterations in type 1 Gaucher disease.[160]

Stage	Skeletal alteration
1	Diffuse osteoporosis
2	Medullary expansion
3	Localised destruction (osteolysis)
4	Ischemic necrosis (long bones); sclerosis; osteitis
5	Diffuse destruction; epiphyseal collapse; osteoarthrosis

The development and progression of bone changes in GD potentially involves a variety of mechanisms (Tab. 4), which are poorly understood.

Infiltration of bone and bone marrow tissues by Gaucher cells is believed to trigger a cascade of reactions.[161] It results in apparently progressive displacement of the fat-rich marrow proper with a consequent shift in haematopoietic activity from proximal to more distal sites.[161] Marrow expansion may cause vascular compression and/or occlusion and increased intraosseous pressure.[161] Localised bone disease, including cortical thinning and long bone deformity, generally occurs in areas adjacent to bone marrow infiltration.[161]

Focal disease, consisting of osteonecrosis and osteosclerosis, is characterised by infarctions caused by Gaucher cells, thrombosis and possibly inflammatory processes.[161]
Generalised osteopenia presumably results from abnormally high rates of bone resorption and reduced rates of bone formation, although osteoclasts and osteoblasts exhibit a normal morphology without lipid accumulation. There is no evidence that Gaucher cells directly erode bone, but they may stimulate bone resorption by secreting specific cytokines and lysosomal enzymes that will interfere with the normal functions of osteoclasts and osteoblasts.[161]

Table 4. Etiologic mechanisms accounting for bone changes in Gaucher Disease.[161]

1.	Progressive accumulation of glucosylceramide
2.	Secretion or induction of cytokines that stimulate bone resorption
3.	Displacement of adipocytes and of hematopoietic elements
4.	Depletion of osteoblasts and osteoclasts precursors
5.	Increased intraosseous pressure, with extravascular compression and occlusion

Although ERT is clearly efficient in reverting most of the symptoms associated with GD, the skeleton response appears to be much slower and attenuated.[122] The reasons behind this are not clearly understood yet. It may be related to an inefficiently delivery of exogenous enzyme to bone;[162] or to its lower effectiveness in the target bone cells.[122, 163] Quantitative analysis of exogenous mannose-terminated GCR uptake in the rat showed that the liver was the main site of uptake (ca. 65% of the dose), and that smaller amounts (less than 3%) were taken up by the spleen and bone marrow.[164] However, the relatively slow skeletal response to enzyme therapy does not necessarily imply poor delivery of enzyme to the skeleton. In fact, tissue γ-scintigraphy distribution studies with tracer doses of radio-iodinated mannose-terminated enzyme in patients with type I GD show avid uptake in the marrow in proportion to the putative cellular pool of macrophages in this organ.[165] Nevertheless, the authors also showed that the enzyme exhibits an apparent shorter half-life in marrow tissue, which may have implications in the treatment, and suggest that frequent administration of subsaturating doses of enzyme may be needed to restore enzyme activity consistently in bone.

Several aspects concerning investigation on GD need further study, but it seems clear that the delayed and modest skeletal response to ERT remains the major issue in the overall effectiveness and management of patients affected by this disease.[122] Apparently, and in particular in the case of adult patients with lesions in an advanced state of progression, skeletal disease is an example of end-organ damage for which adjuvant therapies are necessary to restore bone function more rapidly and/or to a greater extent than with the currently available therapies. It is conceivable to think that an earlier achievement of a complete response would allow an effective establishment of the individually lowest effective dose, and thus a less expensive treatment regimen.

The challenges include the establishment of new strategies to prevent, reverse and control further bone abnormalities, to minimize patient discomfort, maximize function and enhance quality of life.

4. Aim and structure of the thesis

In this thesis, different types of microspheres were developed to be used as injectable materials for bone regeneration and as enzyme delivery systems.
Gaucher disease (GD), a genetic disturbance to which are associated disabling osteoporotic bone lesions, was selected as a model disease, where the use of an injectable material able to enhance bone regeneration and at the same time provide localised enzyme delivery to bone could be beneficial. For this reason, microspheres were analysed as vehicles for the recombinant enzyme glucocerebrosidase (GCR), currently used in the treatment of GD.

The experimental work is presented along eight chapters (Chapters II to IX):

Chapter II describes the use of alginate microspheres as a model-matrix for the entrapment of recombinant GCR. The enzyme was successfully entrapped in alginate microspheres, retaining full activity and exhibiting improved stability at physiological pH. Studies using GCR-deficient fibroblasts from a GD patient showed that released GCR was internalised by cells, significantly enhancing their residual enzymatic activity, with only half of the dose required using free-GCR. The *in vitro* GCR release profile was characterised by a high burst effect followed by a stage of very slow release.
Whether or not this release kinetics is adequate for the therapeutic efficiency of GCR in the *in vivo* targeting of bone resident Gaucher cells needs to be evaluated. However, the possibility of obtaining different patterns of enzyme release increases the range of applications of the system. Moreover, it has been previously reported that attachment-dependent cells are unable to specifically interact with alginate hydrogels, which promote minimal protein adsorption presumably due to their high hydrophilic nature.[166]
So, as a strategy to modulate GCR release-kinetics and at the same time improve cell adhesion to alginate microspheres, calcium phosphate particles were added to the polymeric matrix and calcium phosphate-alginate microspheres were prepared. Two different types of calcium phosphates were used: hydroxyapatite (HAp), a ceramic widely used in orthopaedic applications, and calcium titanium phosphate (CTP), a ceramic that is currently under investigation in our laboratory and has been used as an immobilisation matrix for several enzymes.[167]

In **Chapter III** the preparation and characterisation of calcium phosphate-alginate microspheres is described. The ceramic powders were mixed with alginate and microspheres were prepared by droplet formation under a coaxial air flow, followed by ionotropic gelation of the polymeric matrix in the presence of Ca^{2+}. This resulted in the formation of an alginate hydrogel network with entrapped ceramic particles. The individual components (ceramics and polymer) and the microspheres were characterised using different techniques.

A preliminary evaluation of the microspheres as GCR delivery matrices was also undertaken. The enzyme was incorporated in the microspheres before gel formation in two different ways: pre-adsorbed onto the ceramic particles or directly dispersed in the polymeric/ceramic paste. The two strategies used for GCR entrapment resulted in distinct release profiles. When GCR was pre-adsorbed onto the ceramic powders prior to microspheres formation the initial burst was significantly reduced, compared to the one of alginate microspheres, and a more gradual release was obtained, showing that release-kinetics modulation was achieved.

In **Chapter IV** the adhesion of osteoblastic-like cells to calcium phosphate-alginate microspheres of different compositions was analysed. Results demonstrated that the ceramic-to-polymer ratio strongly influenced the ability of cells to adhere and spread on the microspheres surface. Cells were only able to adopt a flattened morphology on microspheres with a high percentage of ceramic.

In view of these findings, microspheres composed only of calcium phosphate appeared to be suitable for the envisaged application. Calcium phosphate materials have long been recognized as adequate scaffolds for bone regeneration and some are already used in clinical practice. Moreover, they can be more easily sterilised, which is advantageous for both *in vitro* and *in vivo* applications.

For that reason, calcium phosphate microspheres were subsequently prepared and characterised as described in **Chapter V.** The microspheres were obtained by sintering calcium phosphate-alginate microspheres in order to burn-off the polymer and bond together the ceramic granules, while maintaining the spherical-shape of the particles.

In **Chapters VI** and **VII** the behaviour of bone cells cultured on calcium phosphate microspheres was investigated. In **Chapter VI,** the adhesion and proliferation of human osteoblastic-like cells (MG63 cell line) on HAp-microspheres was analysed, while in

Chapter VII a more detailed study on the proliferation, activity and osteogenic differentiation of bone marrow stromal cells cultured on CTP-microspheres is presented. Both materials seem adequate for the culture of bone cells.

The possibility of coupling the enzyme GCR by adsorption to calcium phosphate-microspheres was then considered. Upon adsorption, however, proteins generally undergo structural rearrangements that may result in denaturation and/or loss of functional activity. Nevertheless, these structural rearrangements can sometimes be beneficial rather than detrimental, by leading to enhanced enzyme stability and/or activity. In order to investigate some fundamental aspects of the interaction of recombinant GCR with surfaces, and the suitability of using adsorption as a GCR immobilisation method, some mechanistic studies using highly ordered model surfaces were performed.

In **Chapter VIII** self-assembled monolayers (SAMs) of different functionalities (X= CH_3, OH and COOH) were used to investigate the effect of surface chemistry and solution pH on GCR adsorption to solid surfaces. These studies revealed that the amount and specific activity of adsorbed enzyme were strongly dependent on the conditions used. The results showed that GCR has higher affinity for hydrophobic than for hydrophilic surfaces, and that highest adsorption was obtained at the more acidic pH values, irrespective of the type of surface. However, adsorption on hydrophobic CH_3-SAMs resulted in a decreased specific activity for all the pH tested. Adsorption on gold and on the more hydrophilic SAMs (OH- and COOH-), resulted in different degrees of inactivation at the more acidic pH, and in enzyme activation at higher pH values, near the isoelectric point of the enzyme.

In view of these results, the possibility of using adsorption as a GCR immobilisation method seemed appropriate. In **Chapter IX,** some preliminary results on the ability of calcium phosphate microspheres to reversibly adsorb the enzyme while preserving its biological activity, and to efficiently deliver it into GCR-deficient cells from GD type I patients are presented.

In the last part of this thesis **(Chapter X)**, a short general discussion (concluding remarks) is presented, since a detailed discussion is already provided in each of the preceding chapters. Possible directions for future research (perspectives) are also given.

Most part of the work described in this thesis (Chapters II to VIII) has been published or accepted for publication in international journals.

Papers resulting from this thesis:

1. **Barrias CC**, Granja PL, Meriem L, Sá Miranda MC, Barbosa MA. Biological evaluation of calcium alginate microspheres as a vehicle for the localised delivery of a therapeutic enzyme. Accepted for publication in Journal of Biomedical Materials Research, 2005 **(Chapter II)**.

2. Ribeiro CC, **Barrias CC**, Barbosa MA. Calcium phosphate-alginate microspheres as enzyme delivery matrices. Biomaterials 2004;25:4363-4373 **(Chapter III)**.

3. **Barrias CC**, Ribeiro CC, Rodrigues D, Sá Miranda MC, Barbosa MA. Effect of calcium phosphate addition to alginate microspheres: modulation of enzyme release kinetics and improvement of osteoblastic cell adhesion. Key Engineering Materials 2005;284-286:689-692 **(Chapter IV)**.

4. Ribeiro CC, **Barrias CC**, Barbosa MA. Preparation and characterisation of calcium-phosphate microspheres for biomedical applications. Journal of Materials Science Materials in Medicine 2005, In Press **(Chapter V)**.

5. **Barrias CC**, Ribeiro CC, Barbosa MA. Adhesion and proliferation of human osteoblastic cells seeded on injectable hydroxyapatite microspheres. Key Engineering Materials 2004;254-256:877-880 **(Chapter VI)**.

6. **Barrias CC**, Ribeiro CC, Lamghari M, Sá Miranda MC, Barbosa MA Proliferation, activity and osteogenic differentiation of bone marrow stromal cells cultured on calcium titanium phosphate microspheres. Journal of Biomedical Materials Research A 2005;72A:57-66 **(Chapter VII).**

7. **Barrias CC**, Martins MCL, Sá Miranda MC, Barbosa MA. Adsorption of a therapeutic enzyme to self-assembled monolayers: effect of surface chemistry and solution pH on the amount and activity of adsorbed enzyme. Biomaterials 2005;26:2695-2704 **(Chapter VIII)**.

Contributions of the authors to the papers resulting from this thesis:

Papers 1, 3, 5, 6 and 7: Barrias CC planned and conducted all the experimental work and wrote the manuscripts. However, the interdisciplinary nature of the investigation required the expertise and contribution of the co-authors in the following aspects:

1. Granja PL: assistance in planning the experimental work; Lamghari M: assistance in planning the enzyme uptake studies by Gaucher disease fibroblasts.

3. Ribeiro CC: synthesis of calcium titanium phosphate, assistance in the preparation and characterisation of the microspheres and in enzyme release studies; Rodrigues D: assistance in enzyme activity studies.

5. Ribeiro CC: assistance in the preparation and characterisation of the microspheres.

6. Lamghari M: isolation and culture of rat bone marrow stromal cells; Ribeiro CC: synthesis of calcium titanium phosphate.

7. Martins MCL: assistance in the preparation and characterisation of self-assembled monolayers and in enzyme adsorption studies.

Papers 2 and 4: The work described in these two papers is the result of a strong collaboration between Barrias CC and Ribeiro CC. This partnership has arisen from the interest of the Ribeiro CC in finding a new application for calcium titanium phosphate, namely as an injectable bone-filler material, and the interest of Barrias CC in finding alternative ceramic materials for enzyme immobilisation. In paper 2 Barrias CC assisted Ribeiro CC in the preparation and morphological characterisation of the microspheres and in enzyme immobilisation and release studies. In paper 4 Barrias CC assisted Ribeiro CC in the preparation and morphological characterisation of the microspheres. Barrias CC also participated in the writing of the two manuscripts.

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Chapter II

Biological evaluation of calcium alginate microspheres as a vehicle for the localised delivery of a therapeutic enzyme*

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ABSTRACT

Gaucher disease (GD) is caused by the decreased activity and/or stability of the lysosomal enzyme glucocerebrosidase (GCR). The available treatment consists in the intravenous administration of exogenous GCR, and is effective in reverting most of the symptoms. However, in terms of bone pathology, which is among the most disabling manifestations, a slow and incomplete response is observed, indicating that adjuvant therapies are necessary to consistently restore GCR activity in bone and accelerate regeneration. In this study, calcium alginate microspheres were analysed as a vehicle for localised GCR delivery to bone. Results demonstrated that the entrapped enzyme retained full activity and exhibited a broader pH-dependent activity profile, compared to that of free-GCR, with improved stability at physiological pH. GCR release profile was established, and it was demonstrated that GCR could be released in a sustained manner. The biological behaviour of the system was evaluated by analyzing the uptake of released GCR by GCR-deficient cells from a GD patient, using different techniques: GCR activity measurements, radiolabelling and

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cellulose acetate electrophoresis. Results demonstrated that GCR was internalised by cells significantly enhancing the residual enzymatic activity. To achieve an activity reconstitution level comparable with that obtained using free-GCR, only half of the dose was required with entrapped-GCR.

Keywords: Localised delivery, therapeutic enzyme, calcium alginate, microspheres.

INTRODUCTION

Gaucher disease (GD), the most prevalent lysosomal storage disturbance, is a genetic disorder of lipid metabolism characterised by the markedly decreased catalytic activity and/or stability of the enzyme glucocerebrosidase (GCR), which results in intracellular accumulation of glucosylceramide.[1,2] The occurrence of lipid-engorged cells, preferentially macrophages (Gaucher cells), in a variety of tissues where they replace their normal counterparts, underlies the most common signs of GD, namely: haematological abnormalities, organomegaly, and skeletal disease.[1,2] Considering the non-neuronopathic form of GD (type 1), skeletal lesions are among the most debilitating consequences, having a much greater impact on patient's quality of life than the haematological and visceral abnormalities. Nearly all affected patients show some degree of bone involvement, and skeletal complications are generally accompanied by an increased predisposition to fractures.[1]

The currently available treatment consists in the intravenous administration of recombinant GCR. The modified enzyme exposes mannose residues that increase its affinity for mannose receptors on the target cells, allowing its internalisation and subsequent action *in situ*.[3,4] Although the enzyme replacement therapy (ERT) seems to be efficient in reverting most of the symptoms, the delayed and modest skeletal response remains the major issue in the overall effectiveness and management of affected patients.[1,5,6] This may be related to an inefficient delivery of exogenous enzyme to bone, or to its lower effectiveness in targeting bone Gaucher cells.[1,2] It has been demonstrated that visceral organs avidly uptake most of the injected enzyme diverting it away from other sites affected by the disease.[7] In addition, the apparently shorter half-life of GCR in marrow tissue suggests that frequent administration of subsaturating doses of enzyme may be needed to consistently restore

GCR activity in bone.[7] This is indicative that adjuvant therapies are necessary in order to re-establish bone function more rapidly and/or to a greater extent than with ERT alone.[1]

In the present work, a vehicle for recombinant GCR is proposed. In association with ERT, the present approach could contribute to solve orthopaedic disturbances associated with GD by providing local enzyme delivery to bone lesions associated with higher risks of fracture.

Some attempts to develop GCR carriers for *in vivo* delivery were made in the past, prior to the recognition that macrophages expressed specific receptors for the endocytosis of mannosylated glycoproteins. Entrapment of GCR into different carriers, namely erythrocyte ghosts and liposomes, was then envisaged to exploit the phagocytic capacity of target cells that would account for the internalisation of the exogenous enzyme.[8-11] However, clinical trials were disappointing.[8,10,11] The use of liposomes as GCR carriers was revisited some years ago.[12] High encapsulation efficiencies were reported but, to the authors' knowledge, these studies were not further developed.

In this study, alginate was selected as a model-matrix for the entrapment of GCR, due to its versatility and well known applicability in the biomedical field. Sodium alginate forms relatively stable hydrogels through ionotropic gelation, in the presence of many multivalent ions, being Ca^{2+} the most widely used. The crosslinking process can be carried out under very mild conditions, at low temperature and in the absence of organic solvents, and hydrogels of different shapes can be prepared. Several proteins, including growth factors and enzymes, have already been successfully incorporated in alginate gels, retaining a high percentage of biological activity.[13-18] Moreover, alginate hydrogels have been widely studied for cartilage and bone regeneration applications, as scaffolds and vehicles for biologically active molecules or cells.[19-21]

The main objective of this study was to investigate if recombinant GCR could be entrapped in alginate microspheres crosslinked with Ca^{2+} (Ca-alginate microspheres), while preserving its biological activity, and be efficiently delivered to GCR-deficient cells from type I GD patients.

MATERIALS AND METHODS

Materials

Pharmaceutical-grade sodium alginate with a high α-L-guluronic acid content (65-75%, as reported by the manufacturer) was kindly donated by Pronova Biopolymers and used without further purification. Sodium alginate aqueous solutions were prepared fresh as needed. GCR was purchased from Genzyme Corporation as a lyophilized powder. $Na^{125}I$ and Sephadex columns were purchased from Ammersham Pharmacia Biotech, 4-methylumbelliferyl-β-D-glucopyranoside from Glycosynth and sodium taurocholate from Calbiochem. Cell culture reagents were purchased from Gibco, bicinchoninic acid (BCA) assay kit from Pierce, and cellulose acetate sheets (Cellogel) were obtained from Chemtron. Additional chemicals were purchased from Sigma. All solutions were prepared using distilled-deionised water.

Preparation of Ca-alginate microspheres loaded with GCR

Ca-alginate microspheres loaded with GCR were prepared at room temperature, under aseptic conditions, after filter-sterilising (0.22 μm) all the reagents, including the alginate solution. ^{125}I-labelled GCR, prepared according to the Iodogen method,[22] was used as a tracer. ^{125}I-GCR was purified in a pre-packed PD-10 Sephadex G-25 M column previously blocked with 20% w/v bovine serum albumin (BSA) and equilibrated in phosphate-buffered saline (PBS, pH 7.4). The final solution contained only trace amounts of free ^{125}I (<2%), as estimated by trichloroacetic acid precipitation (TCA, 10% v/v).

^{125}I-GCR was combined with non-labelled GCR (10^7 cpm/mg) immediately prior to use, and dispersed in 3% w/v Na-alginate to a final concentration of 0.2 mg/mL. The solution was extruded drop-wise through a syringe fitted with a 25G needle into a 0.1 M $CaCl_2$ bath (pH 5.6), where microspheres were allowed to harden for 30 min. The obtained GCR-loaded microspheres were recovered and rinsed with saline (0.9% w/v NaCl). The gelling/washing solutions were counted for radioactivity in a γ-counter to estimate enzyme losses during the entrapment process. Microspheres were imaged using a digital camera and their diameter was estimated using a digital calliper (n=20). Different batches of

microspheres were prepared under the same conditions to test the reproducibility of the process.

Characterisation of GCR-loaded Ca-alginate microspheres: loading and catalytic activity yields

To determine the total amount of GCR initially present in freshly prepared microspheres, these were individually counted for radioactivity and the average value calculated (n=20). The catalytic activity of entrapped-GCR was also assayed at different pH values (in the range 4.0 to 7.0), using the assay described in detail below. GCR in solution (free-GCR) was used as a reference. Five replicates were used. The percentage of retained activity (activity yield) was calculated as the ratio of specific activity (activity per unit mass) of entrapped-GCR to that of free enzyme, at the respective pH optimums.

GCR activity assay

The catalytic activity of GCR was assayed as the hydrolysis of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-Glc), as previously described.[23] The final assay mixture contained 5 mM 4-MU-Glc and 6 mg/mL of sodium taurocholate in 50-100 mM citrate-phosphate buffer (pH 5.5, unless otherwise stated). The reaction was allowed to proceed for 30 min in a humidified atmosphere at 37ºC and then stopped with 1 M glycine in 30% w/v NaOH (pH 10.3). The product 4-methylumbelliferone (4-MU) was quantified by fluorescence spectroscopy with excitation at 366 nm and emission at 445 nm, using commercial 4-MU as a standard.

GCR release studies

Release studies were performed using cell culture medium as the test solution. The medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v foetal bovine serum, 1.5% v/v Ultraser G, 2.5 μg/mL fungizone, 200 U-200 μg/mL peniciline-streptomicine and 50 μg/mL kanamicine (complete culture medium). Samples (n=3 batches of microspheres) were maintained under static conditions in a humidified

atmosphere of 5% v/v CO_2 in air. Aliquots (n=5 per batch) from the supernatants were obtained at pre-defined time intervals and counted for radioactivity or assayed for enzymatic activity after dilution in 0.1% v/v Triton X-100 with 1% w/v BSA. Fresh medium was then added to maintain a constant volume. Microspheres were recovered at the end of the release study, rinsed with saline and counted for residual radioactivity. The rinsing solution was also counted to estimate enzyme losses during washing.

Cell cultures

Human fibroblasts from healthy subjects and from type I GD patients (GCR-deficient cells) were obtained from skin explants under consent of the local Ethics Committee, and cultured as described previously.[23] Cells were routinely maintained in complete culture medium at 37ºC in a humidified atmosphere of 5% v/v CO_2 in air. The medium was renewed every 3 days. For subculture, cells were rinsed twice with PBS and harvested with 0.25% w/v trypsin-0.05% w/v ethylenediaminetetraacetic acid (EDTA). Finally, cells were centrifuged, resuspended in fresh complete culture and seeded in 6-well culture plates at a density of 2×10^4 cells/cm^2.

Uptake of GCR by GD fibroblasts

To establish the amount of enzyme that must be added to the medium in order to obtain an acceptable level of GCR activity inside the cells, and to analyse if GCR internalisation increases in a dose-dependent manner, cells were treated with different concentrations of free-GCR (0-50 µg/mL). Intracellular GCR activity was determined after a period of 24 h.
After that, the uptake of GCR by GD fibroblasts treated for 24 h with a defined amount of free- or entrapped-GCR (35 µg/mL, n=3) was evaluated.
A similar study was performed using ^{125}I-GCR as a tracer. In this case, the cell extracts and all the washing solutions were counted for radioactivity. The microspheres were recovered at the end of the assay, rinsed in saline and counted for residual radioactivity.
In both assays, untreated cells from healthy subjects and from type I GD patients were used as references and cell extracts were prepared as described in detail below.

Preparation of cell extracts

After treatment, cells were rinsed twice with warm PBS and harvested with 0.25% w/v trypsin-0.05% w/v EDTA. After centrifugation, supernatants were discarded and cells were washed three times with ice-cold PBS and extracted by brief sonication in 0.1% v/v Triton X-100. The homogenates were centrifuged (5 min, 10,000 rpm) to remove cell debris, and the supernatants (extracts) were used in total protein quantification, GCR activity measurements, and cellulose acetate electrophoresis. Enzymatic activities were determined as described above and normalised to total cell protein content, as measured by the BCA assay using BSA as a standard.[24]

Cellulose acetate electrophoresis

Extracts from control fibroblasts and from GD fibroblasts, before and after treatment with free- or entrapped-GCR, were subjected to cellulose-acetate gel electrophoresis as previously described.[25] This technique is commonly used to separate enzymes in the native state primarily by charge, and bands are only visible if those enzymes are active. Briefly, samples were applied on cellulose-acetate sheets previously equilibrated in the electrophoretic buffer, which consisted of 30 mM sodium phosphate, containing 20% v/v ethylene glycol and 0.5% v/v Triton X-100. The sheets were placed in the electrophoretic tank at 4ºC, and a constant voltage (240 V) was applied for 2.5 h. GCR activity bands were stained by incubation at 37ºC for 1.5 h with 10 mM 4-MU-Glc, 0.2% w/v sodium taurocholate and 0.12% v/v Triton X-100 in 60 mM citrate-phosphate buffer (pH 4). The reaction was stopped with 1M glycine in 30% w/v NaOH (pH 10.3), and the fluorescent bands were visualised at 366 nm and photographed with a type 667 Polaroid film.

RESULTS

Preparation and characterisation of Ca-alginate microspheres loaded with GCR

The entrapment of GCR in Ca-alginate microspheres was performed using ^{125}I-GCR as a tracer. This study allowed the determination of the process efficiency, i.e., the percentage

of enzyme losses during the gelling/washing processes and the final GCR loading. During gelling and washing 35.6±9.9% and 3.1±0.8% of the enzyme was lost, respectively, resulting in a final loading of 73.2±9.4 μg per batch of microspheres (ca. 1.3 μg/microsphere). As shown in Fig. 1, the obtained microspheres were spherically-shaped and presented a uniform size, with an average diameter of 2.4±0.1 mm.

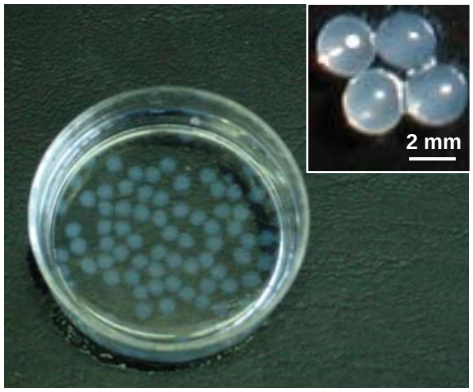

Figure 1. Images of Ca-alginate microspheres loaded with GCR illustrating their spherical shape and uniform size.

The catalytic activity of entrapped-GCR was also characterised. Fig. 2 shows the pH activity profiles of free- and entrapped-GCR. Results are expressed as relative activities, with 100% referring to the activity at the pH optimum for each case. The entrapped enzyme exhibited a broader pH-dependent activity profile, characterised by a greater retention of activity at higher pH values, namely near neutrality. Moreover, the pH optimum was shifted towards the alkaline region by approximately 0.5 pH units.

The activity yield, calculated as the percentage of specific activity of entrapped GCR in relation to that of free enzyme, at the respective pH optimums, was 101±14%.

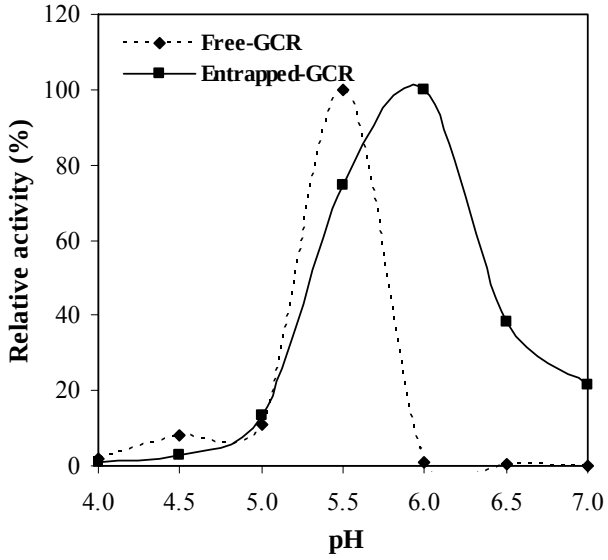

Figure 2. Influence of pH on the activity of free-GCR and entrapped-GCR.

GCR release studies

^{125}I-GCR release from Ca-alginate microspheres at 37ºC in complete cell culture medium, the same conditions used in the uptake study, is presented in Fig. 3. The release profile was characterised by an initial burst, followed by a phase of very slow release. After 24 h, the concentration of GCR in the medium was 15.0±1.4 μg/mL, which corresponds to approximately 40% of the total amount of enzyme initially present in the microspheres.

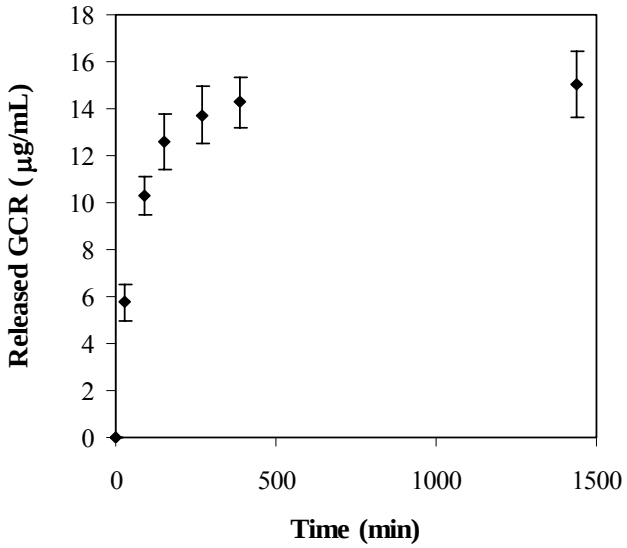

Figure 3. Release profile of GCR from Ca-alginate microspheres. Microspheres were incubated at 37ºC in complete cell culture medium and maintained under static conditions.

Uptake of GCR by GD fibroblasts

Uptake of free-GCR at different concentrations by GD fibroblasts is presented in Fig. 4. By increasing the amount of available GCR in the medium up to 50 μg/mL, the level of GCR intracellular activity increases in a dose-dependent manner, without reaching saturation. GD fibroblasts were then treated with a defined amount (35 μg/mL) of free- or entrapped-GCR. In Fig. 5, results are presented as the activity of GCR in cells, after an incubation period of 24 h. The endogenous activities of GCR in fibroblasts from healthy patients and from GD fibroblasts are also presented as references. In untreated GD fibroblasts, the activity of endogenous GCR is only residual, as expected. However, after treatment with free- or entrapped-GCR, the activity of GCR in GD fibroblasts was significantly raised, approximately 26-fold in both cases, demonstrating that the enzyme was internalised and

that, by comparison with the endogenous GCR activity in fibroblasts from healthy patients, an important activity reconstitution level was achieved.

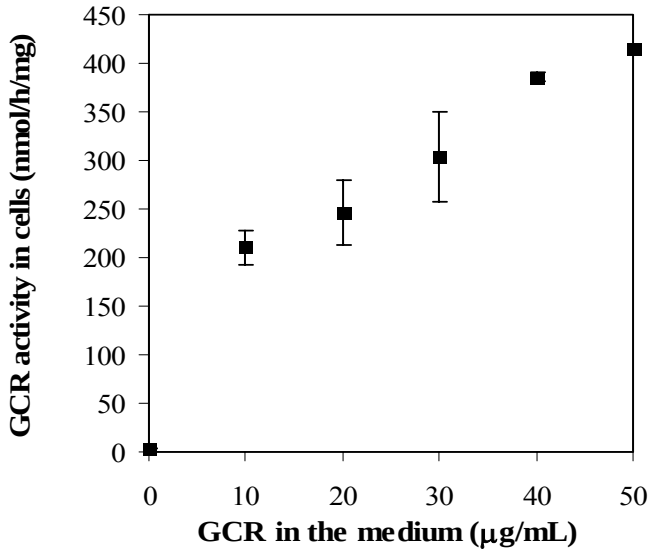

Figure 4. Uptake of free-GCR by GD fibroblasts as a function of enzyme concentration in the medium. Cells were incubated at 37ºC for 24 h with 0-50 μg/mL of free-GCR.

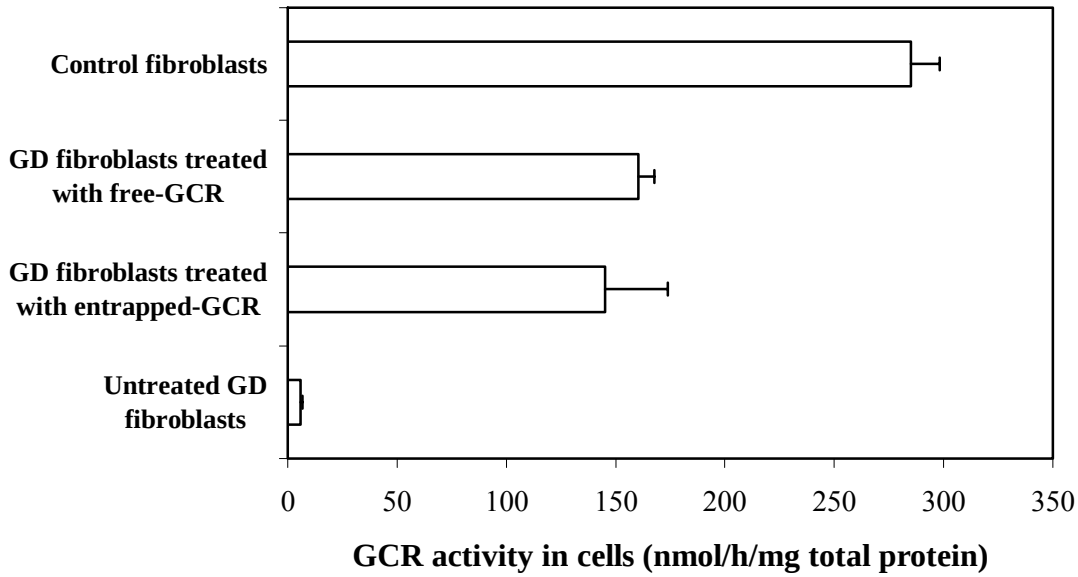

Figure 5. Activity of GCR in GD fibroblasts after treatment with free- or entrapped-GCR, normalised to the total cell protein content. GCR activity in untreated fibroblasts from healthy subjects and from type I GD patients were used as references.

Uptake studies performed with ^{125}I-GCR yielded similar results. Fig. 6a shows that after a period of 24 h, the amounts of enzyme internalised by GCR-deficient cells were approximately the same, whether cells were treated with free- or entrapped-GCR. However, as shown in Fig. 6b, although cells have been treated with the same initial quantity of free- or entrapped-GCR, the amounts of enzyme available in the medium in the two cases were distinct, since only 40% of the entrapped enzyme was released into the medium during the first 24 h, which is in accordance with the results already presented concerning the GCR released studies. Thus, both assays (GCR activity and radiolabelling) indicate that, in order to achieve an enzyme uptake comparable with that obtained using free-GCR, only half of the dose was required with entrapped-GCR.

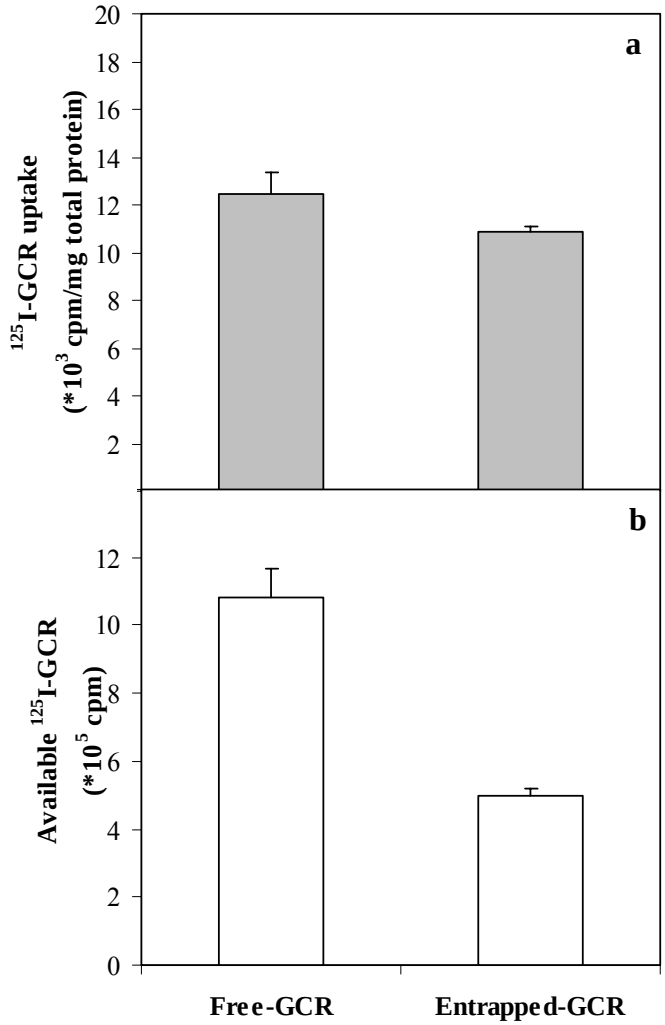

Figure 6. Uptake of free-GCR and entrapped-GCR by GD fibroblasts studied using ^{125}I-GCR as a tracer: (a) Total ^{125}I-GCR internalised by the cells normalised to the total cell protein content, and (b) Total ^{125}I-GCR available in the medium.

Fig. 7 shows the electrophoretic patterns of GCR activity in extracts from control fibroblasts (lane a), from GD fibroblasts treated with free- or entrapped-GCR (lanes b and c, respectively) and from untreated GD fibroblasts (lane d). As expected, GCR activity was not present in the extract obtained from untreated GD fibroblasts. In all other samples, two distinct activity bands (designated band I and II), which represent different physiological forms of GCR, were distinguishable. Band I corresponds to the monomeric form of the enzyme, while band II corresponds to an aggregated form.[25] These results qualitatively confirm the internalisation of both free- and entrapped-GCR by GD fibroblasts, evidencing its integrity in both cases.

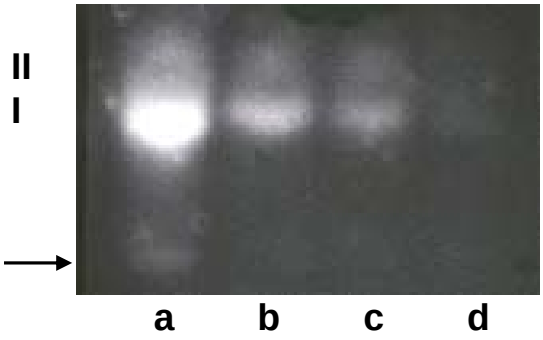

Figure 7. Electrophoretic patterns of GCR activity in extracts from: control fibroblasts (lane a), GD fibroblasts treated with free-GCR (lane b), GD fibroblasts treated with entrapped-GCR (lane c) and untreated GD fibroblasts (lane d). Bands I and II correspond to different physiological forms of the enzyme (monomeric and aggregated, respectively). The arrow points to the site were samples were loaded.

DISCUSSION

In the present study, the application of Ca-alginate microspheres as a vehicle for the localised delivery of recombinant glucocerebrosidase (GCR) was analysed. If successful, this strategy could be used for the therapeutic enhancement of skeletal repair in Gaucher disease. In addition, besides reducing the overall systemic exposure of exogenous GCR, a carrier such as the one proposed may be able to protect the enzyme from *in vivo*

degradation and prolong its biological half-life, thus increasing its therapeutic efficiency.

GCR entrapment in Ca-alginate microspheres was performed using ^{125}I-GCR as a tracer. Microspheres were prepared at pH 5.6, which is below the isoelectric point of the enzyme (in the range of 7 to 8), where it presents a net positive charge, thus theoretically favouring its interaction with the negatively charged carboxyl groups of the polyanionic alginate matrix (pKa of uronic acid residues ≈3.5).[26] Enzyme losses during the entrapment process (ca. 36%) were in agreement with those reported by other authors,[14] and are mainly related to the partially unhardened state of the microspheres during the initial gelling period, where they exhibit increased permeability. The obtained microspheres presented a spherical shape and a uniform size. Although for practical reasons large microspheres were used in this study, smaller ones (down to 400 μm) can be easily prepared in a similar way, by applying a coaxial air stream to pull out the alginate droplets into the crosslinking bath.

Freshly prepared microspheres were characterised in terms of total GCR loading and catalytic activity at different pH values. Entrapped GCR retained full biological activity, suggesting that its native conformation was preserved during the entrapment process. Moreover, the entrapped enzyme exhibited a broader pH-dependent activity profile, compared to that of the free enzyme, with improved stability at more physiological pH values, and a pH optimum slightly shifted towards the alkaline region. This effect has already been reported by other authors[16] and has been attributed to the presence of negatively charged carboxyl groups on the alginate matrix that may attract protons from the bulk solution, thus creating a more acidic local microenvironment.

GCR release from Ca-alginate microspheres was monitored under similar conditions to those used in the cell culture studies, i.e., using complete cell culture medium as the test solution and performing the assay under static conditions for 24 h. The release kinetics appeared to be initially controlled by the diffusion of GCR from the surface of the microspheres (burst effect), followed by diffusion through the pores of the gel matrix. Whether or not this release profile is adequate for the therapeutic efficiency of GCR in the *in vivo* targeting of bone resident Gaucher cells needs to be evaluated. Although out of the scope of this study, modulation of drug release kinetics from hydrogel microspheres has been reported using different strategies, including the application of additional crosslinking or external coatings of polycations, as well as conjugation of the drug with a different carrier, in water-soluble or particulate form, prior to its entrapment.[17,27,28]

The biological behaviour of the system was characterised through short-term cell culture studies, using glucocerebrosidase-deficient fibroblasts from GD patients as a model. Although not being the relevant target cells, fibroblasts are also able to internalise recombinant GCR, through yet unknown mechanisms, and thus are a suitable model for evaluating the integrity of the released enzyme. To analyse the cellular uptake of GCR, preliminary studies were carried out, and dose response was investigated. Subsequently, GD fibroblasts were treated with the same amount of free or entrapped-GCR and enzyme uptake was analysed after 24 h of incubation. The intracellular activity of GCR in treated cells was then compared to that of the endogenous enzyme in fibroblasts from healthy subjects and GD patients. The two quantitative techniques used (GCR activity measurements and radiolabelling) showed that similar activity reconstitution levels were obtained with the two approaches (free- and entrapped-GCR), even if the amount of GCR released from Ca-alginate microspheres was lower (less than 50%) than the total amount of free-GCR present in the medium. This suggests not only that entrapped-GCR maintained its bioactivity, but also that it can be even more effective than the free enzyme. Internalisation of free- and entrapped-GCR was further confirmed qualitatively by cellulose acetate gel electrophoresis. Taken together, these results suggest that the alginate matrix provides a favourable microenvironment to the enzyme and potentiate its biological effect.

CONCLUSIONS

In the present study, a strategy for the therapeutic enhancement of skeletal repair in Gaucher disease via the localised delivery of exogenous glucocerebrosidase is proposed. The enzyme was successfully incorporated in Ca-alginate microspheres, retaining its biological activity. The biological behaviour of the system was evaluated by analysing the uptake of free- and entrapped enzyme by GCR-deficient fibroblasts from GD patients. Results demonstrated that both formulations were internalised by cells and, more importantly, that in order to achieve an activity reconstitution level comparable to that obtained using free-GCR, only half of the dose was required with entrapped-GCR, which suggests that entrapment in the alginate matrix potentiates its biological effect.

Acknowledgements

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Chapter III

Calcium phosphate-alginate microspheres as enzyme delivery matrices*

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ABSTRACT

The present study concerns the preparation and initial characterisation of novel calcium titanium phosphate-alginate (CTP-alginate) and hydroxyapatite-alginate (HAp-alginate) microspheres, which are intended to be used as enzyme delivery matrices and bone regeneration templates. Microspheres were prepared using different concentrations of polymer solution (1% and 3% w/v) and different ceramic-to-polymer solution ratios (0.1, 0.2 and 0.4 w/w). Ceramic powders were characterised using X-ray diffraction, laser granulometry, Brunauer, Emmel and Teller (BET) method for the determination of surface area, zeta potential and Fourier transform infrared spectroscopy (FTIR). Alginate was characterised using high performance size exclusion chromatography. The methodology followed in this investigation enabled the preparation of homogeneous microspheres with a uniform size. Studies on the immobilisation and release of the therapeutic enzyme glucocerebrosidase, employed in the treatment of Gaucher disease, were also performed. The enzyme was incorporated into the ceramic-alginate matrix before gel formation in two different ways: pre-adsorbed onto the ceramic particles or dispersed in the polymeric matrix. The two strategies resulted in distinct release profiles. Slow release was obtained after adsorption of the enzyme to the ceramic powders, prior to preparation of the microspheres. An initial fast release was achieved when the enzyme and the ceramic particles were

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dispersed in the alginate solution before producing the microspheres. The latter profile is very similar to that of alginate microspheres. The different patterns of enzyme release increase the range of possible applications of the system investigated in this work.

Keywords: microspheres, enzyme-delivery, alginate, hydroxyapatite, calcium titanium phosphate.

INTRODUCTION

Osseous tumours, trauma and other debilitating diseases can create a need to fill defects in the skeleton. Most bone tissue engineering strategies rely on the use of temporary scaffolds that can be seeded with cells prior to implantation, or designed to induce the formation of bone from the surrounding tissue after implantation.[1,2] The effectiveness of such materials can be highly improved if they can simultaneously act as drug delivery systems. Depending on the specificity of the illness, bioactive agents (e.g. growth factors or other protein-drugs) can be locally released and potentially accelerate the process of bone regeneration.

In the past few years, increasing efforts have been devoted to the development of improved injectable materials aimed at providing an alternative for the filling of bone defects with less patient discomfort, as they can be applied through minimally invasive surgical procedures. Most injectable materials described in the literature consist of pastes, gels or liquid precursors that solidify *in situ* in response to some stimulus.[3] Micro- or nano-particles have also been described, but they must be suspended in either autologous blood or other appropriate vehicle prior to injection.

A variety of injectable materials, both ceramic- and polymer-based, have been developed for use in multiple orthopaedic applications.[4-20] The combination of ceramic particles with polymeric matrices has also been extensively investigated, in an attempt to mimic bone tissue, which may itself be seen as a complex composite material made of organic and inorganic components. Different ceramic phases have been used, hydroxyapatite and tricalcium phosphate being the most common,[4-9] as well as several polymeric matrices, both from synthetic[10-14] or natural origin, the latter including collagen, chitosan, gelatine and alginate, among others.[15-20]

This investigation describes the preparation and initial characterisation of novel calcium titanium phosphate-alginate (CTP-alginate) and hydroxyapatite-alginate (HAp-alginate)

microspheres intended to be used as injectable enzyme delivery matrices and bone filling materials.

CTP is a bioactive ceramic currently under investigation in our laboratory.[21] Its properties, namely the capacity of ion exchange and chemical adsorption,[22] and the ability to act as an immobilisation matrix for several enzymes,[23] suggest that it can successfully be used in the biomedical field. Furthermore, recent *in vivo* studies showed direct bone contact of implanted cylinders containing calcium titanium phosphate as the main phase.[24] HAp, which has long been recognised for its bioactivity and osteoconductive properties and has been extensively tested as matrix in drug delivery applications,[25,26] was also used in the present investigation.

Alginate was chosen as the polymeric vehicle due to its useful properties and versatility. Ultra-pure grade alginates are considered biocompatible and biodegradable and have been widely used in many biomedical applications, not only as vehicles for biologically active molecules or cells, but also as scaffolds for tissue engineering, either as porous structures or modified with RGD-containing peptide sequences.[27-29] Sodium alginate and most other alginates from monovalent metals are soluble in water, forming solutions of considerable viscosity. Due to their suitable rheological properties, alginates have long been used in the pharmaceutical industry as thickening or gelling agents, as colloidal stabilisers and as blood expanders.[30]

CTP-alginate and HAp-alginate microspheres were prepared using the droplet extrusion method. The ceramic granules were mixed with alginate enabling the preparation of spherical particles through instantaneous crosslinking in the presence of calcium ions.[30] Compared to other methods of preparation of ceramic-polymer microspheres,[31-33] this process presents the advantage of being simple and of being carried out at room temperature and in the absence of organic solvents, which makes it suitable for enzyme entrapment purposes. Moreover, the spherical particles can be easily recovered, without the need for fastidious washing processes, and present a regular size distribution even without subsequent fractionation by sieving.

Analysis of the ability of these matrices to act as carriers for the enzyme glucocerebrosidase (GCR) was also undertaken. This enzyme is used in the treatment of Gaucher disease (type I), which is characterised by a number of severe disabling symptoms, including bone pathologies.[34] GCR is highly unstable in solution under

physiological conditions.[35] Its immobilisation is currently under investigation in our group to overcome this problem.

MATERIALS AND METHODS

Materials

Calcium titanium phosphate [CTP, $CaTi_4(PO_4)_6$] was synthesised by solid state reaction as described elsewhere.[21] Commercial hydroxyapatite (HAp) powder (CAM Implants) pre-heated at 1000ºC was used as a reference. Pharmaceutical-grade sodium alginate (Protanal 10/60 LS) with a high α-L-guluronic acid content (65-75%, as specified by the manufacturer) was kindly donated by Pronova Biopolymers and used without further purification. Na-alginate solutions were prepared fresh as needed, using distilled-deionised water. Purified recombinant human glucocerebrosidase (GCR) was purchased from Genzyme Corporation as a lyophilised powder. $Na^{125}I$ and Sephadex columns were purchased from Amersham Pharmacia Biotech. Additional chemicals were purchased from Sigma.

Characterisation of CTP and HAp powders: X-ray diffraction, specific surface area, granulometry and zeta potential determination

The ceramic powders were analysed by X-ray diffraction (XRD, Philips PW 1710 diffractometer) and their specific surface area was measured by gas adsorption according to the Brunauer, Emmel and Teller (BET) method. Granulometric analysis was performed using a laser scanner particle size analyser (Coulter Electronics Incorporation). Zeta potential (ZP) of CTP and HAp powders was measured with a Coulter Delsa 440 instrument. ZP was calculated automatically by the instrument based on the Smoluchowski formula:

$$\zeta = 4\pi \frac{\mu\eta}{E}$$

where ζ is the ZP (mV), μ the electrophoretic mobility (μm.cm/V.s), η the viscosity of the fluid, and E the dielectric constant of the fluid. The principle of electrophoresis is that a particle will move in a liquid under the influence of an applied electric field provided its ZP is different from zero. The electrophoretic mobility is proportional to the ZP as shown in the equation above.

The ZP of CTP and HAp powders were determined at several pH values. The powders were dispersed in 10 mM KCl and the pH was adjusted with 0.1 M HCl and 0.1 M KOH.

Characterisation of alginate by HP-SEC

High performance size exclusion chromatography (HP-SEC) was performed at room temperature using a modular system, composed of an isocratic pump (K-1001 Knaeur), a vacuum degasser (K-5002 Knaeur), a viscometer/right angle laser light scattering (RALLS) dual detector (T60 Viscotek), and a refractive index detector (K-5002 Knaeur) operating at the same wavelength as the RALLS detector (670 nm). Separations were performed with a set of PL aquagel-OH mixed columns. The mobile phase consisted of 0.1 M $NaNO_3$ with 0.02% w/v NaN_3 and the flow-rate was maintained at 1.0 mL/min. Samples were dissolved in the mobile phase, filtered and injected through a manual injection valve equipped with a 116 μL loop.

Preparation of CTP-alginate and HAp-alginate microspheres

CTP or HAp powders were dispersed in a pre-filtered (0.8 μm) Na-alginate solution under gentle stirring until a homogeneous paste was obtained. Different concentrations of the polymer solution (1% and 3% w/v), and different ceramic-to-polymer solution ratios (0.1, 0.2 and 0.4 w/w) were tested. These will be designated as 10/1, 20/1, 40/1 (ceramic-to-polymer solution ratios, using the 1% w/v Na-alginate solution) and 10/3, 20/3, 40/3 (ceramic-to-polymer solution ratios, using the 3% w/v Na-alginate solution). The pastes were extruded drop-wise into a 0.1 M $CaCl_2$ crosslinking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min. The size was

controlled by regulating the extrusion flow rate using a syringe pump (Cole-Parmer), and by applying a coaxial air stream (Encapsulation Unit Var J1; Nisco). At completion of the gelling period the microspheres were recovered and rinsed in water in order to remove the excess $CaCl_2$. Finally, they were dried overnight in a vacuum oven at 30ºC. The diameter of the microspheres was measured using an inverted plate microscope (Olympus) equipped with an ocular micrometer with an accuracy of 10 μm.

Characterisation of CTP-alginate and HAp-alginate microspheres: SEM and FT-IR analysis

Morphological characterisation of the microspheres (surface and transversal sections obtained by criofracture in liquid nitrogen) was carried out using scanning electron microscopy (SEM). Samples were sputter coated with gold using a JEOL JFC-100 fine coat ion sputter device, and observed using a JEOL JSM-6301F scanning microscope.
Physicochemical characterisation of the microspheres and their components (CTP, HAp and Ca-alginate) were analysed by Fourier transform infrared spectroscopy (FTIR) using a Perkin Elmer system 2000 spectrometer. The FTIR spectrum of Na-alginate was also obtained and used as a reference. Microspheres were reduced to powder and were analysed as KBr pellets.

Enzyme immobilisation in CTP-alginate and HAp-alginate microspheres

The enzyme was incorporated into the ceramic-alginate microspheres (formulation 20/3) before gel formation in two different ways: pre-adsorbed onto the ceramic particles before mixing with the alginate solution, or dispersed in the polymeric-ceramic paste. Microspheres were subsequently prepared as previously described, without drying. In Fig. 1 a schematic representation of the matrices tested is presented. The matrices A and B were used as controls.

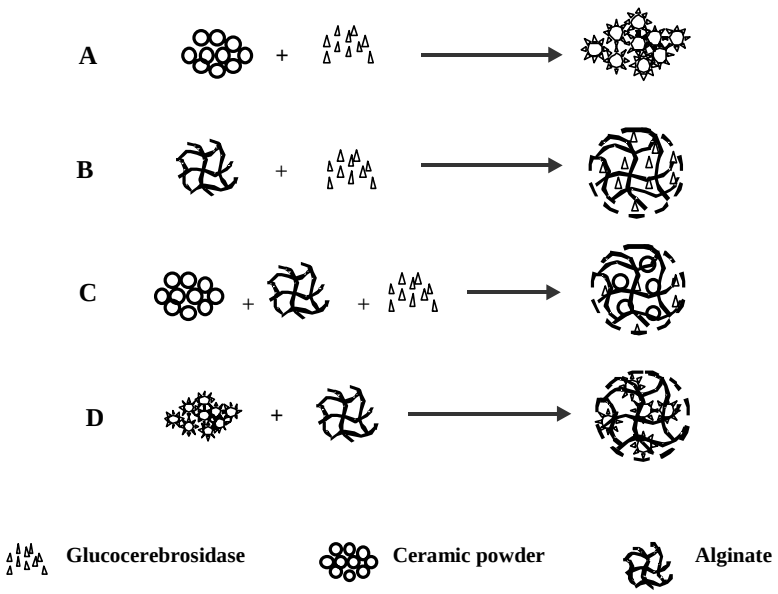

Figure 1. Different enzyme immobilisation matrices tested: (A) enzyme adsorbed onto the ceramic powders, (B) enzyme dispersed in a pure alginate matrix, (C) enzyme and ceramic powders individually dispersed in the alginate matrix and (D) ceramic powders with pre-adsorbed enzyme dispersed in the alginate matrix. The matrices A and B were used as controls.

Pre-adsorption of the enzyme onto CTP and HAp powders

A solution containing glucocerebrosidase (GCR) was obtained by dissolving the enzyme in phosphate-buffered saline (PBS, pH 7.4) at a final concentration of 0.2 mg/mL and in the presence of 0.5 mg/mL of bovine serum albumin (BSA) as a stabiliser. The solution also contained radiolabelled (^{125}I) enzyme (Iodogen method) as a tracer and 0.01 M NaI to competitively reduce binding by any free ^{125}I species present. The specific radioactivity of the solution was 6.5×10^7 cpm/mg GCR.

For adsorption tests, CTP and HAp powders (50 mg) were incubated with 500 μL of the enzyme solution. Samples were maintained at 4ºC in an orbital shaker at 250 rpm. At predefined time intervals, samples were centrifuged (5 min, 14,000 rpm) and the supernatants collected for analysis. The powders were washed twice with PBS and separated by centrifugation. The powders and all the supernatants were counted for radioactivity. The counts of each sample were averaged and surface concentration was calculated by the equation:

$$GCR(ng/cm^2) = \frac{Counts(cpm)}{A_{solution}(cpm/ng) \times SA(cm^2)}$$

where the Counts represent the radioactivity of the powders, the $A_{solution}$ is the specific activity of the protein solution and SA is the surface area of the powders, which was calculated as described previously.

Enzyme release studies

Enzyme release studies were performed in PBS. Samples (n=3) were maintained at 37±0.2ºC in an orbital shaker at 120 rpm. At predefined time intervals, the supernatants were collected and counted for radioactivity and fresh PBS was added. In the case of the powder matrices (Fig. 1, matrix A) samples were centrifuged (5 min, 14,000 rpm) prior to collecting the supernatants. At the end, the matrices were recovered, washed twice with PBS and counted for residual radioactivity.

RESULTS

Characterisation of CTP and HAp powders

X-ray diffraction and specific surface area

X-ray diffraction analysis of the ceramics indicated the presence of mono phase crystalline compounds. The specific surface areas obtained using the BET method were 9.84 cm^2/mg for the CTP powder and 76.00 cm^2/mg for the HAp.

Granulometric analysis

The granulometric analysis of HAp and CTP powders is presented in Figs. 2a and 2b, respectively. The particle size distribution curves of both ceramics are narrow. In the case of the CTP powder, 90% (in volume) of the particles are smaller than 25.32 μm and have a

volume average diameter of 11.00 μm. In the HAp powder 90% of the particles are smaller than 20.51 μm and the volume average diameter is 7.96 μm.

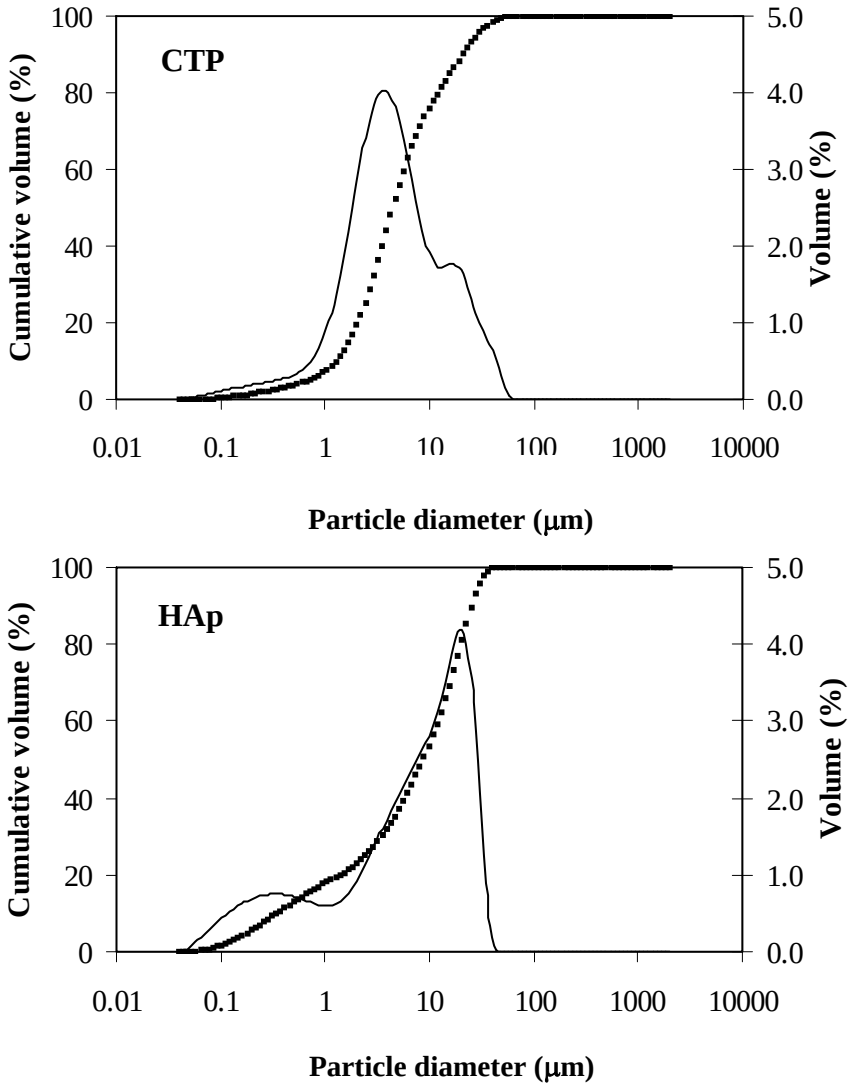

Figure 2. Granulometric analysis of (a) CTP and (b) HAp powders. Cumulative (dashed line) and non-cumulative (solid line) volume percentage particle size distributions are plotted on the left and right axis, respectively.

Zeta potential measurements

ZP measurements of CTP and HAp powders as a function of pH are presented in Fig. 3. As can be observed, the isoelectric point of CTP occurs approximately at pH 3 whereas the isoelectric point of HAp occurs approximately at pH 6. To the best of our knowledge, the

isoelectric point of CTP has not been reported in the literature before. As far as the HAp is concerned, the results obtained are in agreement with those reported by other authors.[36,37] At physiological pH both ceramics are negatively charged, the CTP potential being more negative than that of HAp.

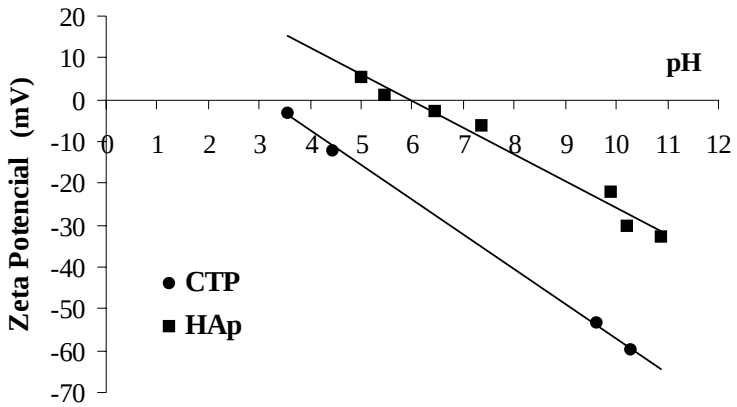

Figure 3. Zeta Potential of CTP and HAp powders as a function of pH.

Characterisation of alginate by HP-SEC

The Na-alginate used in this study was characterised by an average molecular weight (Mw) of 1.3×10^5, a polidispersity index of 1.9 and an intrinsic viscosity of 6.4 dL/g, as determined by HP-SEC.

Characterisation of CTP-alginate and HAp-alginate microspheres

CTP-alginate and HAp-alginate microspheres were prepared using different concentrations of the polymer solution (1% and 3% w/v), and different ceramic-to-polymer solution ratios (0.1, 0.2 and 0.4 w/w). As soon as the ceramic-polymer droplets contacted with the crosslinking solution, spherical-shaped particles were instantaneously formed when using the 3% w/v alginate solution. An exception was observed for the HAp-alginate 40/3 paste, which was too viscous to be extruded in a reproducible manner. Particles obtained using the 1% w/v alginate solution presented a disc-shaped morphology and for that reason were not used in the subsequent experiments.

The diameters of the microspheres obtained (before and after drying) using the 3% w/v polymer solution and the different ceramic-to-polymer ratios are presented in Fig. 4.

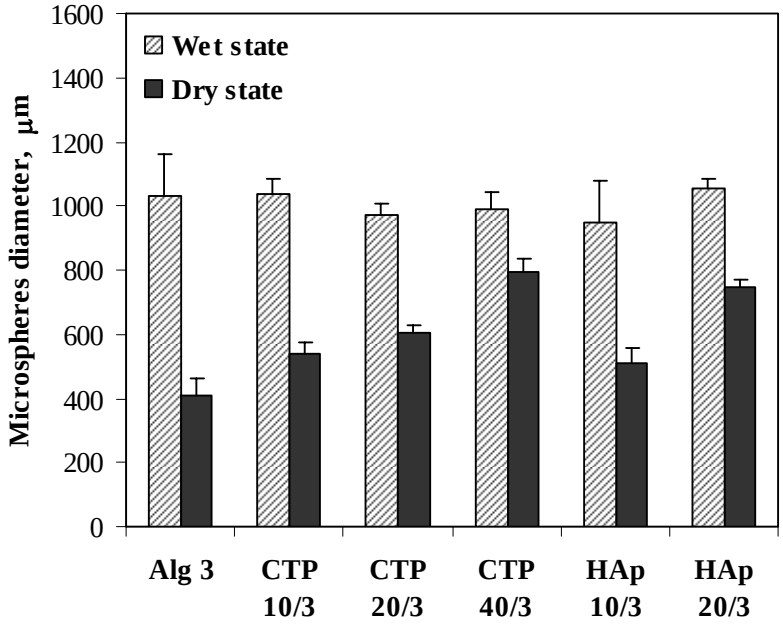

Figure 4. Diameters of alginate, CTP-alginate and HAp-alginate microspheres prepared using the 3% w/v alginate solution, before (wet state) and after drying (dry state).

Before drying, microspheres of approximately 1000 µm were obtained when using pure alginate, CTP with alginate, and HAp with alginate. Upon drying, microspheres have undergone a volume contraction, which was more significant for the lower ceramic-to-polymer solution ratio (0.1 w/w). Microspheres with diameters of 541±32, 606±21, and 796±39 µm were obtained for the CTP 10/3, 20/3 and 40/3 formulations, respectively. When using HAp, microspheres with diameters of 512±44 µm and 749±22 µm were obtained for the 10/3 and 20/3 formulations.

SEM analysis

SEM images of the microspheres (20/3 formulations) are presented in Figs. 5 and 6. Upon drying, and contrary to alginate microspheres that shrank to a great extent and even collapsed (Fig. 5a), CTP-alginate (Fig. 5b) and HAp-alginate (Fig. 5c) microspheres maintained their

original shape, with no evidence of cracks. This suggests that both fillers provided additional control of shrinkage and avoided structural collapse. Different surface roughnesses were obtained, those of the HAp-alginate microspheres being smoother than those of CTP-alginate. The ceramic powders are homogenously distributed in the alginate matrix (Fig. 6) with the granules densely packed and well embedded in the polymer.

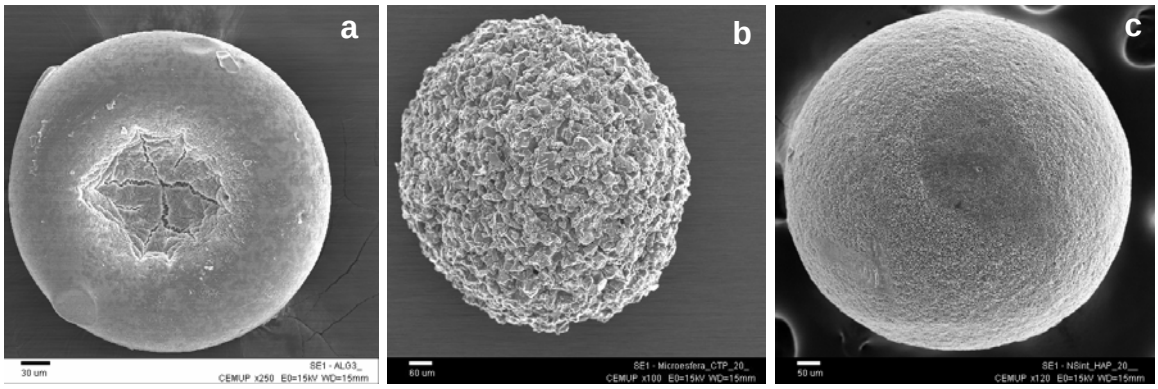

Figure 5. SEM image of microspheres: (a) Ca-alginate, (b) CTP-alginate and (c) HAp-alginate.

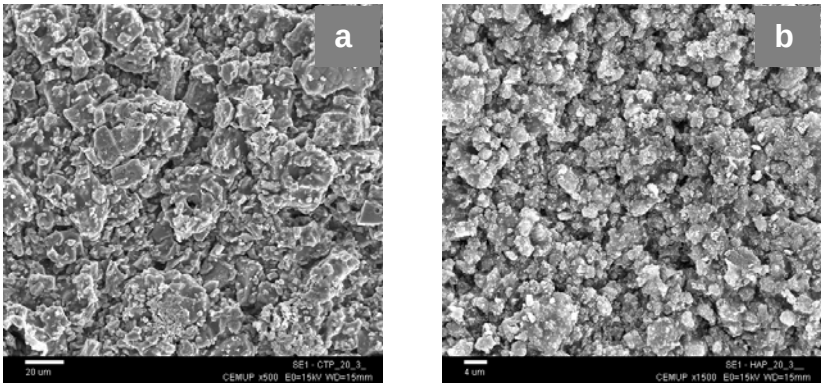

Figure 6. Detail of a transversal section of: (a) CTP-alginate microspheres and (b) HAp-alginate, showing that the ceramic particles are well embedded in the polymer matrix.

FTIR analysis

FTIR spectra of calcium and sodium alginate are represented in Fig 7. Tab. 1 shows the possible assignments for the FTIR bands of the alginate salts.[38] Both spectra are very similar and only slight differences can be observed in the width and height of the COO^- bands.

Fig. 8 shows the FTIR spectra of CTP powder, Ca-alginate and CTP-alginate microspheres (formulations 10/3 and 40/3). The FTIR spectra of HAp powder, Ca-alginate and HAp-alginate microspheres (formulations 10/3 and 40/3) are presented in Fig. 9. The characteristic bands of both ceramics are maintained in the microspheres indicating that the alginate did not induce subsequent modifications in the ceramics structure. Additional bands can be observed, corresponding to the presence of calcium alginate, namely at 3446 cm^{-1} (νOH), 1619 and 1428 cm^{-1} (νCOO^-), and 820 cm^{-1}, identified in the literature as the combination of three possible vibrational modes (τCO+δCCO+δCCH).[38] The intensity of these bands increases as the ceramic-to-polymer solution ratio decreases. Also, the ν_3PO_4 region (900-1200 cm^{-1}) becomes broader in the spectra of the microspheres, denoting the presence of the polymer.

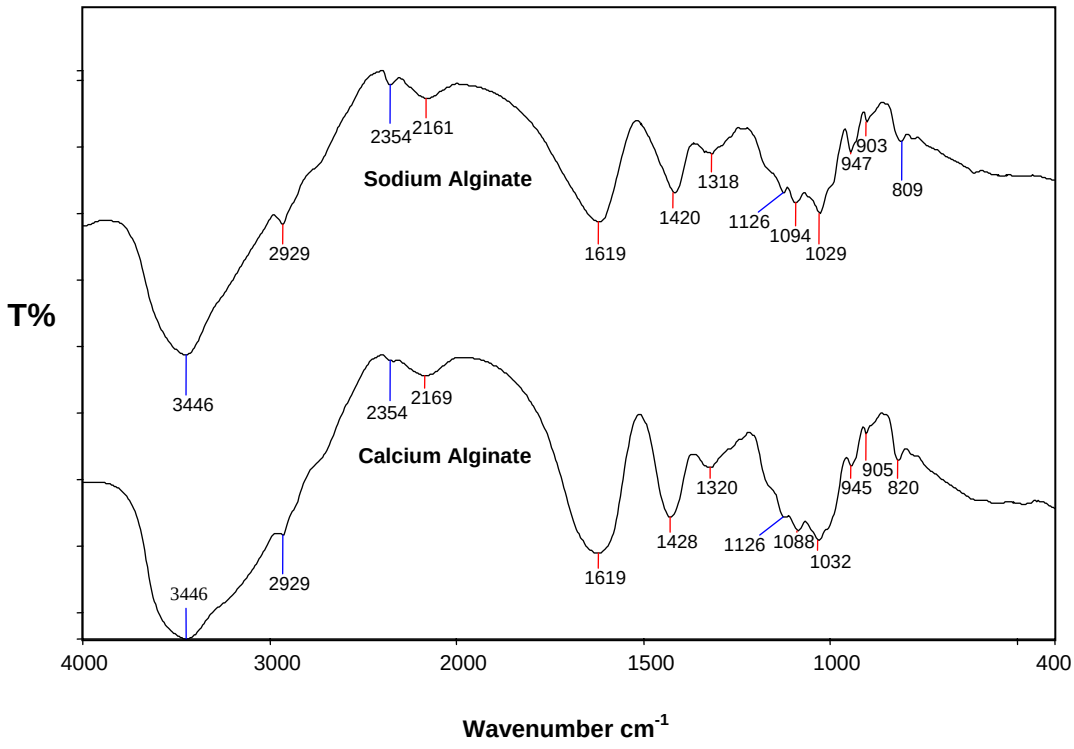

Figure 7. Transmittance (T%, arbitrary units) FTIR spectra of sodium and calcium alginate.

Table 1. Peak assignment of transmittance bands of Na-alginate and Ca-alginate FTIR spectra

Na/Ca-alginate bands (cm^{-1})	Peak Assignment[36]	Na/Ca-alginate bands (cm^{-1})	Peak Assignment[36]
3446	ν (OH) hydrogen bonded	1126	ν (CC), ν (CO)
2929	ν (CH)	1088/1094	τ (CO), δ (CCO), δ (CC)
2354	-	1029/1032	τ (CO), δ (CCO), δ (CC)
2161/2169	-	947/945	ν (CO), δ (CCH)
1619	ν (COO^-)	903/905	ν (CO), δ (CCH)
1420/1428	ν (COO^-)	809/820	τ (CO), δ (CCO), δ (CCH)
1318/1320	ν (COO^-)		

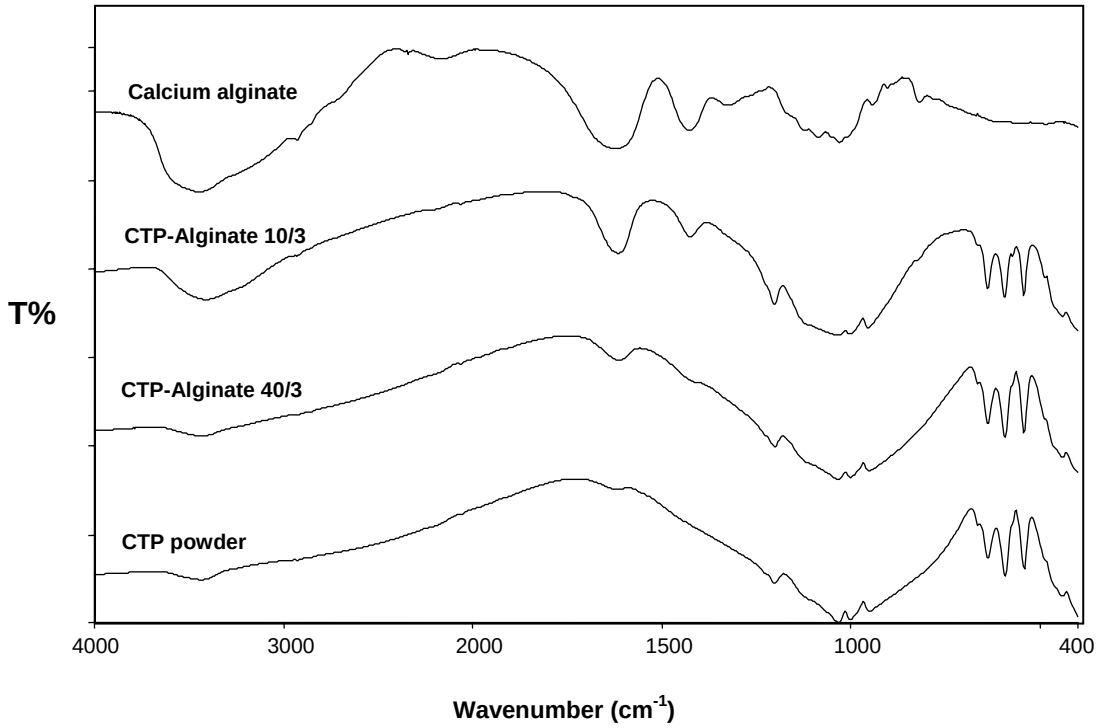

Figure 8. Transmittance (T%, arbitrary units) FTIR spectra of Ca-alginate, CTP-alginate 10/3 and 40/3 microspheres, and CTP powder.

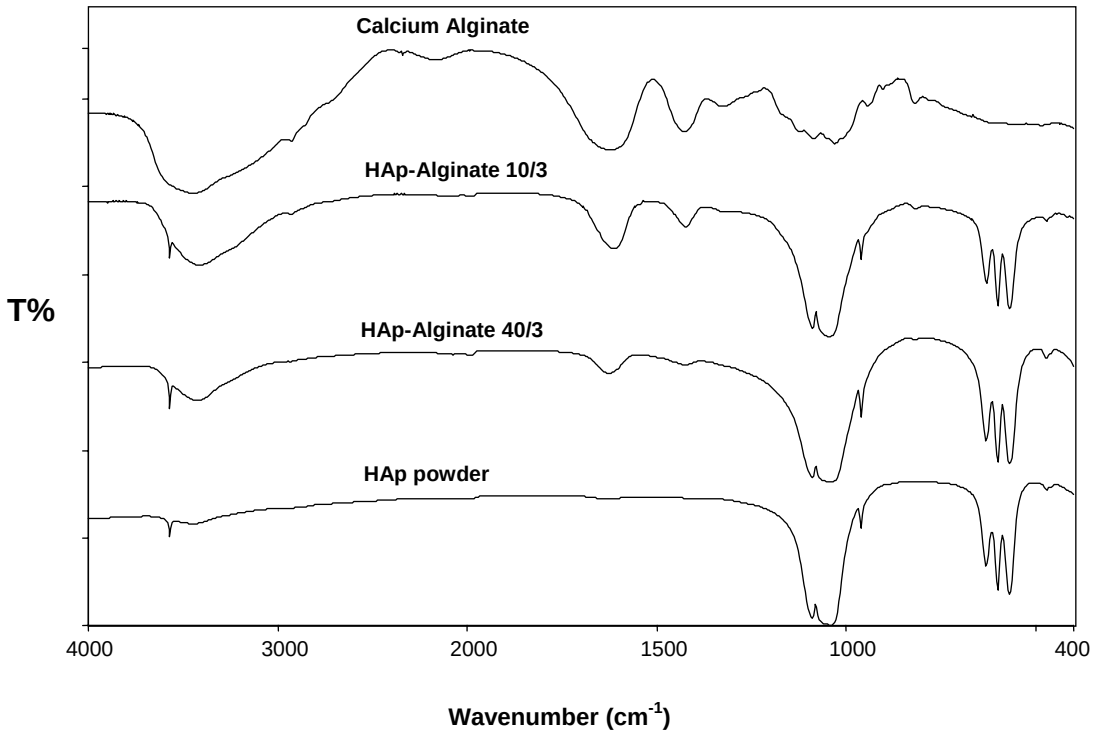

Figure 9. Transmittance (T%, arbitrary units) FTIR spectra of Ca-alginate, HAp-alginate 10/3 and 40/3 microspheres, and HAp powder.

Enzyme immobilisation in CTP-alginate and HAp-alginate microspheres

Pre-adsorption of the enzyme onto CTP and HAp powders

Fig. 10 shows typical adsorption time profiles of GCR onto CTP and HAp powders. The adsorption process was carried out at 4°C for different periods of time (10, 120 and 1440 min). For both ceramics there is an initial period of rapid adsorption followed by a slower approach to a limiting value. After 1440 min, CTP adsorbed 41.28 ng GCR/cm^2, a much higher amount of enzyme per unit surface are than HAp (1.94 ng GCR/cm^2).

Fig. 11 represents the percentage of enzyme, with respect to the total amount used, which becomes adsorbed to the ceramic powders (21% in the case of CTP and 10% in the case of HAp), as well as the percentage of enzyme that remains in solution after incubation. The percentage of enzyme removed from the solids during washing (1st and 2nd washes) is also represented. The percentages of 21% and 10% were measured after washing, indicating that the enzyme remained well adsorbed onto the powders.

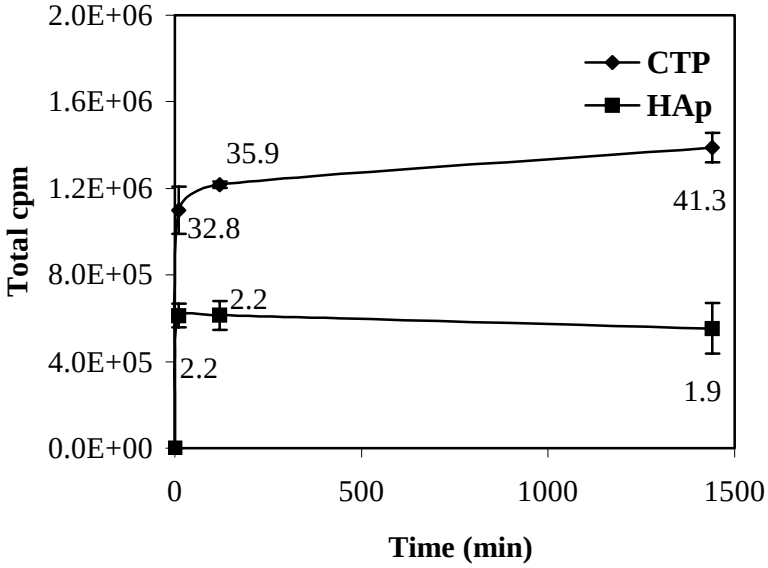

Figure 10. Time profiles of glucocerebrosidase adsorption onto CTP and HAp powders. The adsorption process was carried out at 4°C for different periods of time (10, 120 and 1440 min). Data labels represent the amount of adsorbed protein per unit surface area (ng/cm^2) of the powders.

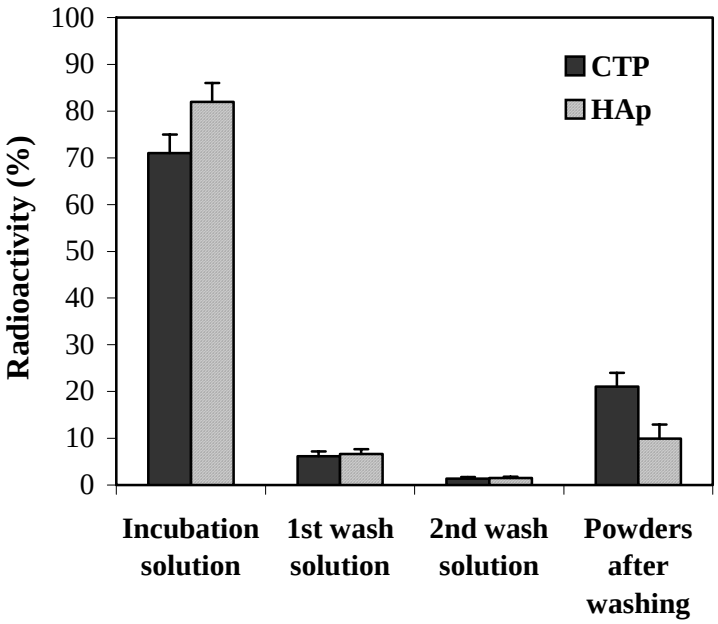

Figure 11. Adsorption of glucocerebrosidase onto CTP and HAp powders: percentage of radioactivity that becomes associated to the powders after an incubation period of 10 min (4°C). The percentages of radioactivity that remain in the supernatant and are washed out from the powders are also depicted.

Enzyme release studies

Fig. 12 shows the release profiles obtained for the different matrices previously described using CTP as the ceramic phase. The release profiles using HAp were similar (results not shown). When the enzyme was dispersed in the ceramic-alginate mixture before gel formation (matrix C), the release profile seems to be initially controlled by the diffusion of GCR from the surface of the beads, with a burst of 33.6±3.3% of total loading, followed by diffusion through the pores of the gel matrix until reaching a plateau at 55.4±4.6%. The overall release profile was not significantly different from what was obtained using a pure alginate matrix (matrix B). When the enzyme was pre-adsorbed onto the ceramic powders prior to the preparation of the microspheres (matrix D), the initial burst was significantly reduced (9.4±1.4%) and a slower release profile was obtained. The results obtained when using matrix A were similar to those reported for matrix D.

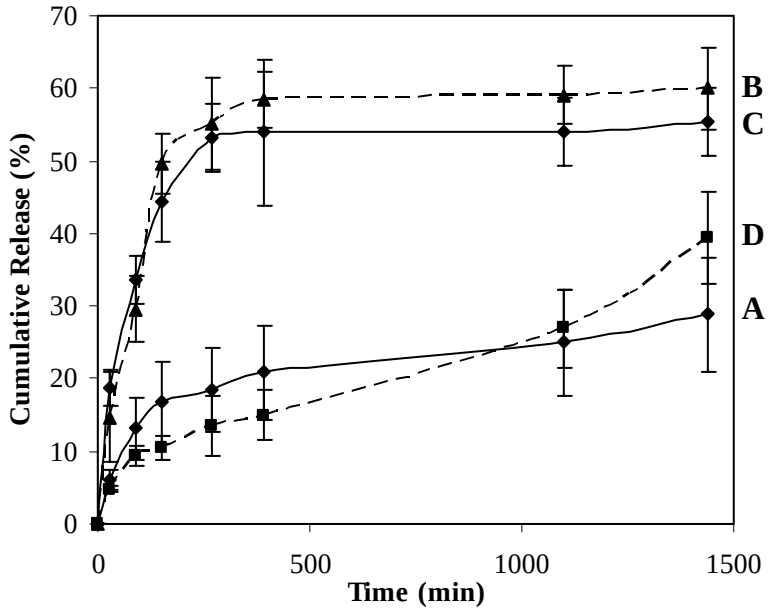

Figure 12. Enzyme release profiles from the matrices tested using CTP: (A) enzyme adsorbed onto the ceramic powders; (B) enzyme dispersed in a pure alginate matrix; (C) enzyme and ceramic powders individually dispersed in the alginate matrix; and (D) ceramic powders with pre-adsorbed enzyme dispersed in the alginate matrix. The matrices A and B were used as controls.

DISCUSSION

CTP-alginate and HAp-alginate microspheres were prepared using the droplet extrusion method combined with ionotropic gel formation in the presence of Ca^{2+}.[27] Polymer-ceramic mixtures of different compositions were prepared and drop-wise extruded into a crosslinking solution containing Ca^{2+}. As soon as the droplets contacted with the solution, spherical particles were instantaneously formed, due to the rapid establishment of calcium-mediated associations between poly-guluronic acid sequences on the polymer backbone.[27] This resulted in the formation of an alginate hydrogel network with entrapped ceramic particles.

The process was carried out at room temperature and in the absence of organic solvents, which is ideal for the envisaged applications of these materials as enzyme carriers.

The described methodology enabled the preparation of homogeneous microspheres presenting a regular size distribution, even without being fractioned by sieving. During the drying process and depending on their composition, microspheres underwent volume contractions at different extents. Shrinkage is related to water loss from the polymeric hydrogel phase, explaining why formulations with high ceramic-to-polymer contents underwent less contraction.

SEM observation of the microspheres showed that the ceramic particles were well embedded and homogeneously distributed in the alginate matrix, suggesting a good wettability of the ceramics by the polymer solution. The surface morphology of the microspheres was dependent on the type of ceramic used, the HAp-alginate microspheres being smoother than the CTP-alginate ones. Differences were essentially attributed to the different mean particle sizes of the two ceramic powders. Although the granulometric analysis revealed that the particles of both powders were similar in size (90% of CTP particles are smaller than 25.32 μm and have an average diameter of 11.00 μm, while 90% of the HAp particles are smaller than 20.51 μm and the average diameter is 7.96), the surface area measurements indicated that HAp particles have a much higher surface area (76 cm^2/mg) than CTP (9.8 cm^2/mg).

Physicochemical characterisation of CTP-alginate and HAp-alginate was carried out by FTIR spectroscopy. The spectra showed that the characteristic bands of both ceramics are maintained in the microspheres, indicating that the alginate did not induce subsequent modifications in the structure of the ceramics.

A preliminary analysis of the ability of CTP-alginate and HAp-alginate microspheres to act

as carriers for the enzyme glucocerebrosidase (GCR), a therapeutic enzyme used in the treatment of Gaucher disease, was carried out.

The possibility of exploiting the capacity of both components to act as enzyme delivery systems was investigated, since both may immobilise proteins.[23-27] For this reason, the enzyme was incorporated into the ceramic-alginate matrix before gel formation in two different ways, as described before. Concerning GCR loading capabilities of the ceramics used in this study, results showed that CTP adsorbs 41.28 ng/cm^2, a much higher amount of enzyme per unit surface than HAp (1.94 ng/cm^2). This may be attributed to the presence of BSA, used to stabilise the enzyme, as explained below. At physiological pH, both ceramics are negatively charged, the zeta potential of CTP being more negative than that of HAp. At pH 7.4, the enzyme is in the vicinity of its isoelectric point exhibiting a neutral net charge. Under the conditions used, BSA presents a net negative charge[39] and will probably bind to a lesser extent to CTP than to HAp, due to increased repulsive charge, thus leaving the surface more available for GCR adsorption. Decreased adsorption of HSA (human serum albumin) onto CTP than onto HAp powders was observed by the authors (unpublished data), supporting this hypothesis. Despite being a very complex process, protein adsorption generally reflects hydrophobic/hydrophilic or electrostatic interactions between the protein and the surface. It seems that under the conditions used, the electrostatic characteristics of CTP favours the immobilisation of GCR.

Other authors, in studies concerning applications in the biotechnology industry,[22] demonstrated the capacity of CTP to immobilise several enzymes. To the best of our knowledge this is the first time that this ceramic is used for the immobilisation of glucocerebrosidase.

Release studies were performed with different matrices. When the enzyme was dispersed in the ceramic-alginate mixture before gel formation (matrix C), the release profile seemed to be initially controlled by the diffusion of GCR from the surface of the beads, with a significant burst, followed by diffusion through the pores of the gel matrix until reaching a plateau. The overall release profile was not significantly different from what was obtained using a pure alginate matrix (matrix B), suggesting that the enzyme does not interact, to a great extent, with the embedded ceramic particles. When the enzyme was pre-adsorbed onto the ceramic powders prior to the preparation of the microspheres (matrix D) the initial burst was significantly reduced and a slower release rate was achieved. For matrix A (powders)

the results were similar, suggesting that the alginate does not offer an additional resistance to enzyme diffusion.

With the different matrices proposed, distinct release kinetics can be obtained and suitable strategies can be selected depending on the final application. Some aspects remain, however, to be investigated, namely the activity of the enzyme when released from the matrices used in this work (ongoing studies). Although protein immobilisation, either by entrapment or adsorption, often results in conformational alterations that may render the protein inactive, several authors have reported improved enzyme activity and/or stability in immobilised preparations when compared to their free forms.[40]

CONCLUSIONS

This study describes the preparation and initial characterisation of CTP-alginate and HAp-alginate microspheres, which are intended to be used as enzyme delivery matrices and bone regeneration templates. The proposed methodology enabled the preparation of homogeneous microspheres with a uniform size, where the bulk properties of the ceramics were maintained, indicating that the alginate did not induce any modifications in the structure of the ceramics. Preliminary studies on the immobilisation and release of the therapeutic protein glucocerebrosidase were also performed. The enzyme was incorporated into the ceramic-alginate matrix before gel formation in two different ways: pre-adsorbed onto the ceramic particles or dispersed in the matrix. The two strategies resulted in distinct release profiles, suggesting that, depending on the application, the more suitable one can be selected.

Acknowledgements

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Chapter IV

Effect of calcium phosphate addition to alginate microspheres: modulation of enzyme release kinetics and improvement of cell adhesion*

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ABSTRACT

In this study, the addition of calcium phosphate powders to an alginate matrix was evaluated as a strategy to modulate enzyme release-kinetics from alginate microspheres and, simultaneously, to improve cell adhesion to the polymer. Pre-adsorption of the enzyme to the ceramic powders resulted in a more adequate release pattern. The ratio of ceramic-to-polymer had a pronounced effect on osteoblast adhesion to microspheres. Cells were only able to spread on microspheres with the highest percentage of ceramic (0.4 w/w using a 1.5% w/v alginate solution).

Keywords: hydroxyapatite, calcium titanium phosphate, alginate, microspheres, enzyme release, cell adhesion

INTRODUCTION

The activity of the enzyme glucocerebrosidase (GCR) is deficient in type I Gaucher disease (GD), a genetic disturbance that characteristically results in severe haematological

abnormalities, organomegaly and skeletal deterioration.[1] The currently available treatment is based on the intravenous administration of exogenous enzyme, and it seems to be efficient in reverting most of the symptoms.[1] However, in terms of bone pathology, which is among the most disabling manifestations of GD (type 1), a slow and incomplete response to the treatment is observed, even when high doses are used, indicating that adjuvant therapies are necessary in order to consistently restore enzyme activity in bone.[1] An injectable vehicle for local GCR-delivery to bone is currently under investigation. GCR was previously entrapped in alginate microspheres, retaining full activity and exhibiting improved stability at physiological pH. Studies using GCR-deficient fibroblasts from a GD patient showed that released GCR was internalised by cells, significantly enhancing their residual enzymatic activity, with only one half of the dose required using free-GCR. Release profiles were characterised by a high burst effect followed by a stage of very slow release. As a strategy to modulate GCR release-kinetics and at the same time improve osteoblastic cell adhesion to alginate microspheres, calcium phosphate powders of hydroxyapatite (HAp) or calcium titanium phosphate (CTP) were added to the polymeric matrix and ceramic-alginate microspheres were prepared by droplet extrusion followed by ionotropic gelation with Ca^{2+}. In this study, GCR release kinetics and osteoblast adhesion to ceramic-alginate microspheres of different compositions were analysed.

MATERIALS AND METHODS

Preparation and characterisation of HAp-alginate and CTP-alginate microspheres

HAp or CTP powders were mixed with Na-alginate solution (1.5 and 3% w/v) at different ratios (0.1, 0.2 and 0.4 w/w). The different formulations are designated in the text as 40/1.5 (0.4 w/w ceramic using 1.5% w/v Na-alginate) and 10/3, 20/3 and 40/3 (0.1 to 0.4 w/w ceramic, using 3% w/v Na-alginate). The paste was extruded drop-wise into a $CaCl_2$ bath, where microspheres were instantaneously formed. Size was controlled by applying a coaxial air stream. After 30 min, microspheres were recovered and rinsed in water. Characterisation was carried out by FTIR and SEM. Diameters were measured using an optical microscope with an ocular micrometer.

GCR entrapment in HAp-alginate and CTP-alginate microspheres and release studies

Radiolabelled enzyme (^{125}I-GCR, Iodogen method) was incorporated in the polymeric/ceramic paste prior to extrusion in two different ways: directly dispersed in the paste or pre-adsorbed onto the ceramic particles. Ceramic-alginate microspheres with entrapped GCR were then prepared as already described. Alginate microspheres with entrapped GCR were used as controls. For enzyme adsorption, the ceramic powders were immersed in 0.1 mg/mL GCR solution in phosphate-buffered saline (PBS, pH 7.4) and incubated at room temperature for 30 min under agitation. A control GCR solution at 0.1 mg/mL was incubated under the same conditions (without powders) to account for eventual enzyme losses due to adsorption to the tubes walls. At the end, powders were washed in PBS and separated by centrifugation. To determine the amount of adsorbed GCR, the concentration of enzyme in the supernatants before and after adsorption was quantified by the bicinchoninic acid method. GCR activity in the supernatants and in the powders was assayed as the hydrolysis of the substrate 4-methylumbelliferyl-β-D-glucopyranoside (5 mM) in 50-100 mM citrate-phosphate buffer (pH 5.5) and in the absence of activators. GCR release studies from the different matrices were performed in PBS at 37ºC and 120 rpm. At predefined time intervals, aliquots from the supernatants were collected and counted for radioactivity and fresh PBS was added to maintain a constant volume.

Cell adhesion studies

Microspheres were pre-incubated in 70% v/v ethanol, equilibrated overnight in α-minimal essential medium supplemented with 10% v/v foetal bovine serum, and finally seeded with osteoblast-like MG63 cells. Alginate and HAp microspheres were used as controls. After 24 h, microsphere-cell constructs were visualised by SEM and by confocal laser scanning microscopy (CLSM). For CLSM, f-actin filaments were stained with Alexafluor 488-conjugated phalloidin (Molecular Probes) and nuclei were counterstained with propidium iodide (Sigma).

RESULTS AND DISCUSSION

Preparation and characterisation of HAp-alginate and CTP-alginate microspheres

Spherical-shaped microparticles presenting a uniform size (Fig.1a) were prepared. The diameters of CTP-alginate microspheres ranged from 450±14 μm to 796±39 μm, while the diameters of HAp-alginate microspheres ranged from 412±23 μm to 749±22 μm, both depending on the formulation. SEM images of both types of microspheres revealed that the ceramic particles are well embedded and homogenously distributed in the alginate matrix as illustrated in Fig.1b (20/3 HAp-alginate microspheres). FTIR analysis (data not shown) indicates that the characteristic spectral bands of both ceramics are maintained in the microspheres, in comparison with the starting powders, suggesting that the alginate did not induce subsequent modifications in the ceramics structure.

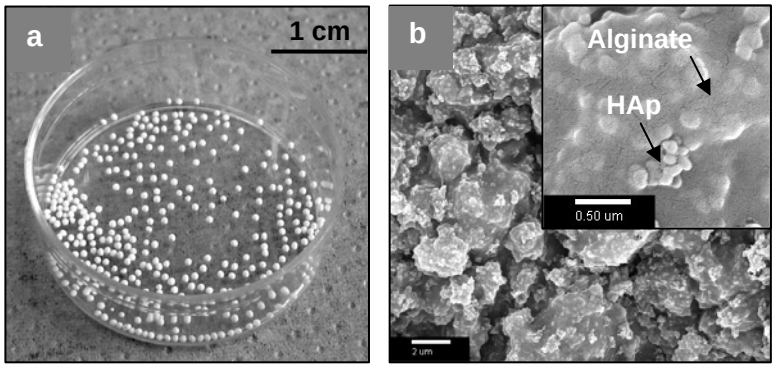

Fig 1. a) Photograph of CTP-alginate microspheres (40/3) illustrating their uniform size; b) SEM of a HAp-alginate microsphere (20/3) showing the ceramic particles embedded in the polymer.

GCR entrapment in HAp-alginate and CTP-alginate microspheres and release studies

The enzyme was incorporated in the ceramic-alginate paste prior to the preparation of the microspheres in two different ways: directly dispersed in the paste or pre-adsorbed onto the ceramic particles. The ceramic powders used were characterised by a specific surface area of 9.8 cm^2/mg (CTP) and 76.0 cm^2/mg (HAp). Adsorption was performed at pH 7.4, which is near the enzyme isoelectric point (in the range 7 to 8) where the enzyme exhibits higher structure stability and therefore a smaller tendency to denaturate upon adsorption. The amount of adsorbed GCR was calculated by quantifying GCR depletion from the

supernatant. Preliminary assays showed that the incubation time selected (30 min) was sufficiently long to attain steady-state adsorption values. GCR has higher affinity for CTP powder, which adsorbed 41.8±1.1 ng GCR/cm^2, than for HAp, which adsorbed 4.3±0.4 ng GCR/cm^2. The variation in plateau values for the two ceramics is probably related to differences in protein-surface electrostatic interactions. Fig. 2a depicts GCR activity in the initial solution (free enzyme only) and in the presence of the two ceramic powders after adsorption (free + adsorbed enzyme) as a function of the GCR amount in the assay. In all cases, GCR activity increases linearly with the amount of enzyme present, but while in the presence of HAp there is some degree of inactivation, in the presence of CTP a higher catalytic efficiency was observed. Differences probably result from GCR conformational alterations in the adsorbed state. The specific activity (activity per unit mass) of GCR adsorbed on CTP and HAp powders after washing and re-suspending in fresh PBS (adsorbed enzyme only) were c.a. 91.5±4.9% and 10.3±0.1% of the control (free enzyme), respectively. These values may however be underestimated due to enzyme losses by desorption during washing.

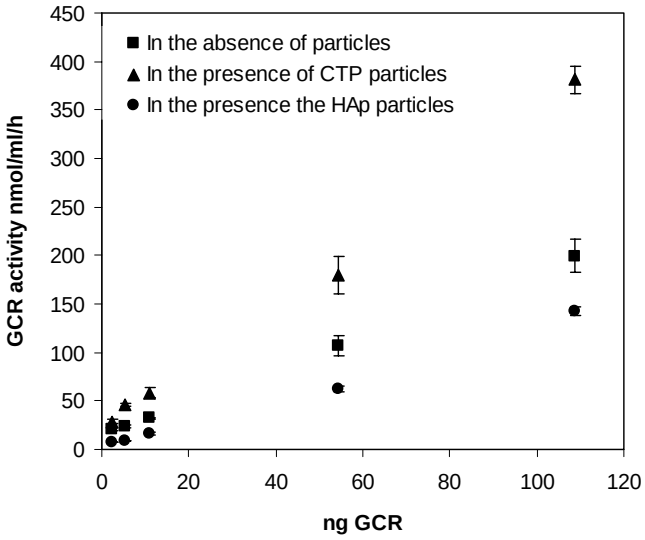

Fig 2a. GCR activity in the absence and in the presence of the two ceramic powders after adsorption, as a function of the GCR amount in the assay.

Release studies

The two strategies used for GCR entrapment in the alginate matrix resulted in distinct release kinetics, but similar results were obtained for both ceramics tested. Fig. 2 shows typical release profiles obtained with HAp-alginate and alginate microspheres. When GCR

was pre-adsorbed onto the ceramic powders prior to microspheres formation the initial burst was significantly reduced, from ca. 50% to 11% (t=150 min) compared to the one of alginate microspheres, and a slower and more linear release profile was obtained, showing that release-kinetics modulation was achieved. The release profile of GCR directly dispersed in the ceramic/polymer matrix was not significantly different from the control (data not shown), suggesting that the GCR does not interact, at least to a great extent, with the embedded ceramic particles.

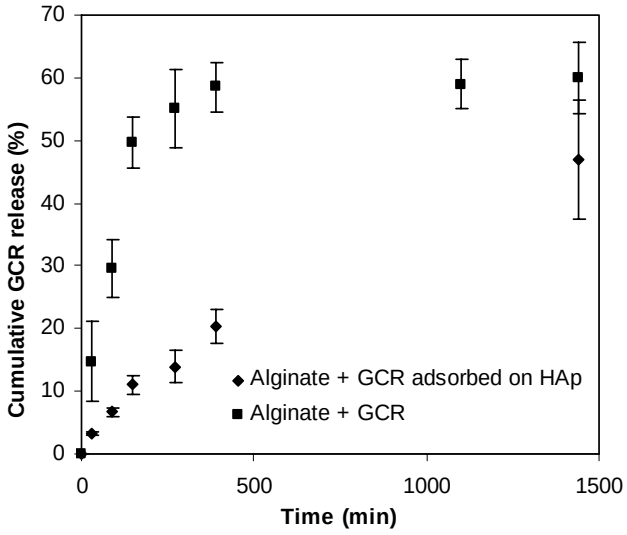

Fig 2b. GCR release from alginate and HAp-alginate microspheres (formulation 20/3).

Cell adhesion studies

The ability of cells to adhere and spread on the surface of HAp-alginate and CTP-alginate microspheres of different compositions was assessed by CLSM (Fig. 3) and SEM (Fig. 4). Figures are referred to HAp-alginate microspheres but similar results were obtained for both ceramics tested. No adherent cells could be found on the surface of control alginate microspheres (data not shown). This was not unexpected since it has been previously reported that attachment-dependent cells are unable to specifically interact with alginate hydrogels, which promote minimal protein adsorption presumably due to their high hydrophilic nature.[2] Upon addition of HAp or CTP particles to the alginate matrix a distinct cell behaviour was observed, depending on the ceramic-to-polymer ratio used. For the lower ceramic-to-polymer ratios (formulations 10/3, 20/3 and 40/3) only dispersed round cells and/or multicellular aggregates could be observed on the microspheres surface

(Fig. 3a, 3b and 4a). However, on microspheres prepared with the higher ceramic-to-polymer ratio (formulation 40/1.5) cells were able to attach, spread and adopt a spindle-shaped morphology (Fig. 3c and 4b). Filopodial extensions could be observed both by CLSM and SEM. Cells on control HAp microspheres were well flattened exhibiting a typical osteoblastic-like morphology (Fig. 3d and 4c). Enhanced cell adhesion in polymer-ceramic composites in comparison with their polymeric counterparts has been previously demonstrated by other authors.[3]

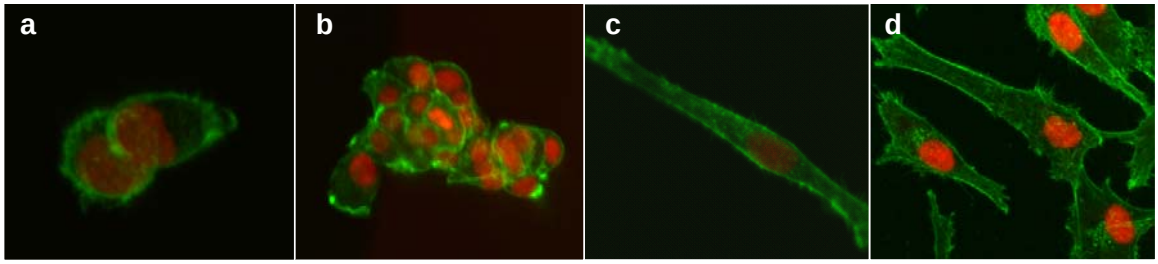

Fig 3. CLSM images of MG63 cells cultured for 24 h on: a) 10/3, b) 20/3, c) 40/1.5 HAp-alginate microspheres and d) HAp microspheres.

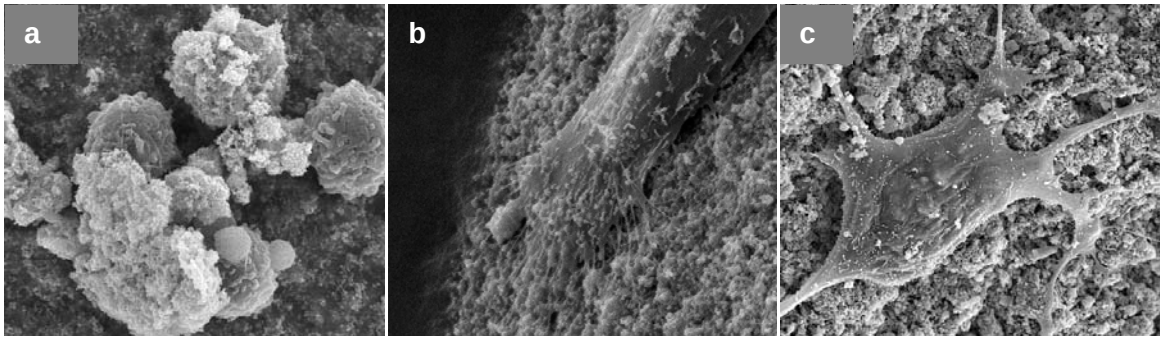

Fig 4. SEM images of MG63 cells cultured for 24 h on: a) 20/3, b) 40/1.5 HAp-alginate microspheres and c) HAp microspheres.

CONCLUSIONS

The addition of calcium-phosphate particles to alginate allowed the modulation of GCR release kinetics. The ratio of ceramic-to-polymer had a pronounced effect on osteoblasts adhesion to microspheres. Cells were only able to spread on microspheres with the higher percentage of ceramic.

Acknowledgments

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Chapter V

Preparation and characterisation of calcium phosphate porous microspheres with a uniform size for biomedical applications*

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ABSTRACT

In the present work, a novel route for the preparation of porous ceramic microspheres is described. Two ceramic powders, calcium titanium phosphate (CTP) and hydroxyapatite (HAp), were mixed with a sodium alginate solution that enabled the preparation of spherical particles, using the droplet extrusion method combined with ionotropic gelation in the presence of Ca^{2+}. The spherical particles were subsequently sintered, to burn-off the polymer and obtain calcium phosphate microspheres with a uniform size and an interconnected porous network. CTP microspheres with diameters ranging from 513±24 μm to 792±35 μm and with pores of approximately 40 μm were obtained. HAp microspheres presented diameters of 429±46 μm and 632±40 μm and pores of ca. 2 μm. Depending on the formulations tested; the structure of both calcium phosphates may become altered during the sintering process, suggesting that the ratio between the ceramic phase and the polymer solution is a critical parameter. Porous microspheres prepared using the described methodology are promising candidates as bone defect fillers and scaffolds for bone tissue regeneration.

Keywords: porous microspheres, calcium titanium phosphate, hydroxyapatite, alginate, bone regeneration.

INTRODUCTION

Due to the large number of orthopaedic surgical interventions performed each year, bone repair has become a subject of intensive investigation. The use of calcium phosphate ceramics in bone regeneration, either alone or in combination with a polymeric phase, has become a common practice, since these materials generally provide good biological responses and adequate mechanical properties. Although calcium phosphate ceramics have traditionally been used as dense or porous three-dimensional scaffolds, increasing efforts have been devoted to the development of injectable formulations for bone-defects filling applications, since they can be applied through minimally invasive techniques.[1] Most injectable ceramic materials described in the literature consist of micro or nanoparticles suspended in appropriate vehicles.[2-7] In such systems, the shape of the microparticles determines their packaging characteristics, spherical particles being more suitable for implantation than non-homogeneous granules, since they conform better to irregular implant sites. Moreover, uniform particles present more predictable flowing properties during injection.[8-11]

In certain applications, the effectiveness of these microparticulate materials can be highly improved if they can act simultaneously as carriers for biologically active molecules. In this sense, porous materials are advantageous, since they present additional surface area, an important parameter that strongly influences the loading capacity and release rates that can be obtained.

In the present work, novel ceramic porous microspheres are proposed, which could find applicability in the field of bone regeneration, both as injectable bone-filling materials and drug delivery matrices. Two ceramic powders, calcium titanium phosphate (CTP) and hydroxyapatite (HAp), were tested. CTP, a bioactive ceramic currently under investigation in our laboratory,[12] is a potential material to be used in the biomedical field as it presents interesting properties, namely the ability to act as an immobilisation matrix for several enzymes.[13-16] HAp, which possesses a chemical structure similar to bone mineral, has long been recognized in the biomedical field for its osteoconductivity and capacity to act as matrix for drug delivery[17-18] and was used as a reference material. Alginate was chosen due to its suitable rheology and ability to form relatively stable hydrogels under mild conditions.[19]

CTP-alginate and HAp-alginate microspheres were first prepared using the droplet extrusion method as described in a previous work.[16] The ceramic powders were mixed with alginate, which enables the preparation of spherical particles through instantaneous crosslinking in the presence of Ca^{2+}.[16] The obtained microspheres were then dried and subsequently sintered to burn-off the polymer and aggregate the ceramic granules, allowing the preparation of ceramic microspheres with a uniform size and interconnected porous network.

Compared to other methods of preparing ceramic microspheres described in the literature,[8-11] this novel process presents the advantage of simplicity and of being carried out in the absence of organic solvents or oils, thus enabling the recovery of the spherical particles without the need of fastidious washing processes. Moreover, the obtained microspheres present a regular size distribution, even without a subsequent fractionation by sieving.

In this study, the preparation and physicochemical characterisation of CTP and HAp microspheres prepared according to the described methodology are presented.

MATERIALS AND METHODS

Characterisation of the ceramic powders

CTP was synthesised by solid-state reaction as described elsewhere.[12] HAp was kindly donated by CAM Implants. Both ceramics were analysed by X-ray diffraction (XRD, Philips PW 1710 diffractometer), and their granulometry characterised using a laser scanner particle size analyser (Coulter Electronics Incorporation). The specific surface area of each powder was measured by gas adsorption according to the BET (Brunauer, Emmel and Teller) method. Both powders were observed by scanning electron microscopy (SEM). Samples were sputter coated with gold using a JEOL JFC-100 fine coat ion sputter device, and observed using a JEOL JSM-6301F scanning microscope.

Preparation of CTP and HAp microspheres

Ceramic powders were dispersed in a pre-filtered (0.8 μm) 3% w/v sodium alginate solution (Pronova Biopolymers) under gentle stirring until a homogeneous paste was obtained.

Different ceramic-to-polymer solution ratios (0.1, 0.2 and 0.4) were tested. These will be designated as 10/3, 20/3 and 40/3 (different ceramic-to-polymer solution ratios, using a 3% w/v sodium alginate solution). Preliminary assays were conducted to determine the maximum ceramic-to-polymer solution ratio and it was observed that for ratios higher than 0.4 the pastes obtained became too viscous to be handled and extruded. The pastes were extruded drop-wise into a 0.1 M $CaCl_2$ crosslinking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min. The size of the microspheres was controlled by regulating the extrusion flow rate using a syringe pump (Cole Parmer) and by applying a coaxial air stream (Encapsulation Unit Var J1; Nisco). At completion of the gelling period, microspheres were recovered and rinsed in water in order to remove the excess $CaCl_2$. Finally, they were dried overnight in a vacuum-oven at 30ºC, and then sintered at 1100ºC with a uniform heating rate of 5ºC/min, and a 1 h stage at the maximum temperature.

Characterisation of the microspheres

The diameter of the microspheres (n=20) was measured using an inverted plate microscope (Olympus) equipped with an ocular micrometer with an accuracy of 10 μm.
Morphological and physicochemical characterisation was carried out using SEM and Fourier transform infrared spectroscopy (FTIR). For FTIR analysis, microspheres were reduced to powder and analysed as KBr pellets using a Perkin Elmer System 2000 spectrometer.
In order to identify the composition of the residue that results from burning the polymer during sintering, calcium alginate microspheres, produced as described, were submitted to the same heating cycle used for preparing CTP and HAp microspheres. The obtained residue was analysed by XRD and FTIR.

RESULTS

Characterisation of the ceramic powders

XRD of the ceramics indicated the presence of monophase crystalline compounds. Granulometric analysis of the powders revealed that 90% of the CTP particles are smaller than 25.32 μm, with a volume average diameter of 11.0 μm, and that 90% of the HAp particles are smaller than 20.51 μm, with a volume average diameter of 7.96 μm. However, surface area measurements indicated that HAp particles have a much higher surface area (76 cm^2/mg) than CTP (9.8 cm^2/mg) suggesting that the results obtained in the HAp granulometric analysis are overestimated, probably due to particle aggregation. Both powders were observed by SEM, which confirmed that CTP particles are in fact much larger than the HAp particles (data not shown).

Characterisation of CTP and HAp microspheres

To produce CTP and HAp microspheres, CTP-alginate and HAp-alginate mixtures of different compositions were prepared and drop-wise extruded into a 0.1M $CaCl_2$ solution. As soon as the ceramic-polymer droplets contacted with the crosslinking bath, spherical particles of approximately 1000 μm were instantaneously formed, due to the rapid establishment of calcium-mediated junctions between poly-guluronate chains on the polymer backbone.[18] An exception was observed for the 40/3 HAp formulation, with which it was not possible to obtain spherical particles due to the high viscosity of the ceramic-polymer mixture.

During drying, the volume contractions varied with the formulation as shown in Fig.1 which presents the diameters of dried CTP and HAp microspheres, that were obtained before (ceramic-polymer microspheres) and after sintering (ceramic microspheres).

It can be concluded that the higher the ceramic-to-polymer solution ratio used, the lower the particles contraction during drying. Upon sintering, microspheres maintained their original spherical shape. In the case of the HAp microspheres their diameter further decreased approximately 20%, while CTP microspheres practically maintained their original dimensions.

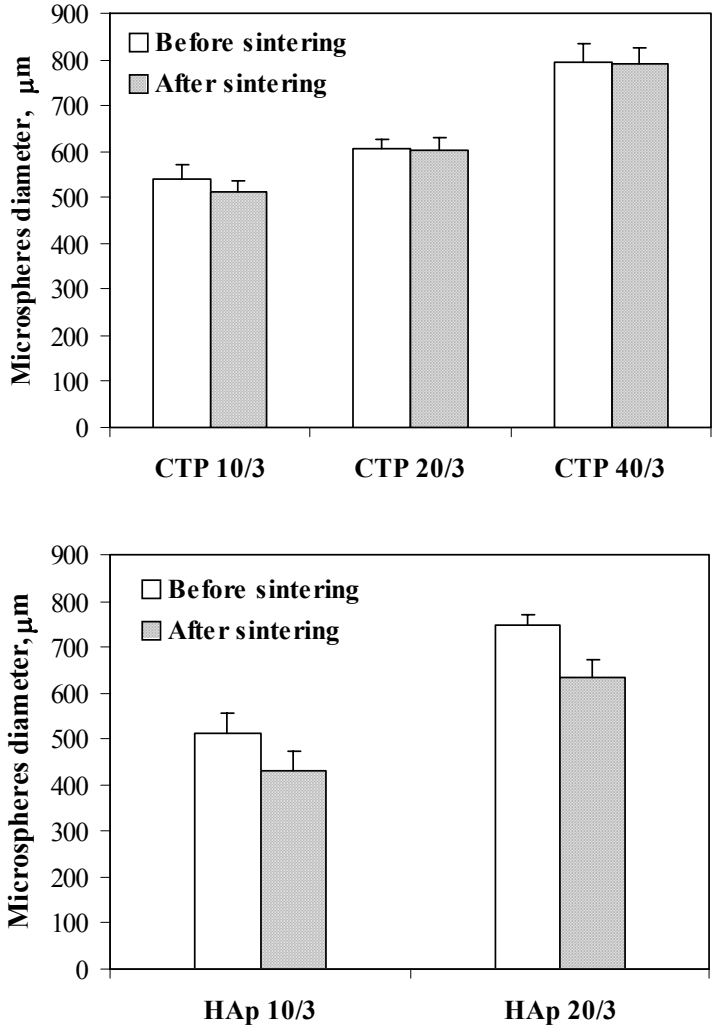

Figure 1. Diameters of CTP microspheres; and HAp microspheres, before and after sintering.

Such differences are probably related to the different particle size distributions and, in particular, to the higher percentage of fine particles in HAp that results in a more effective packaging during sintering. With the described methodology, microspheres with a regular size distribution were obtained without being necessary a subsequent fractioning by sieving. Microspheres with average final diameters of 513±24 μm, 602±28 μm, and 792±35 μm were obtained for the CTP 10/3, 20/3 and 40/3 formulations, respectively; while with HAp microspheres diameters of 429±46 μm and 632±40 μm were obtained for the 10/3 and 20/3 formulations, respectively.

SEM analysis

SEM pictures of CTP and HAp microspheres, before and after sintering, are presented in Fig. 2. Only the results obtained with the 20/3 formulation are given, as similar results were obtained for the other formulations tested. The analysis revealed that in both cases microspheres were homogeneous, in terms of size and shape. No evidence of cracks was found. CTP microspheres (Fig. 2 a, b, c) present a rather rough surface, while HAp microspheres (Fig. 2 d, e, f) are smoother. Such differences are mainly attributable to the differences in granulometry of the two ceramics, since the CTP particles are much larger than those of HAp. The volume contraction that occurred upon sintering in the case of HAp microspheres is evident (Fig. 2 a, b).

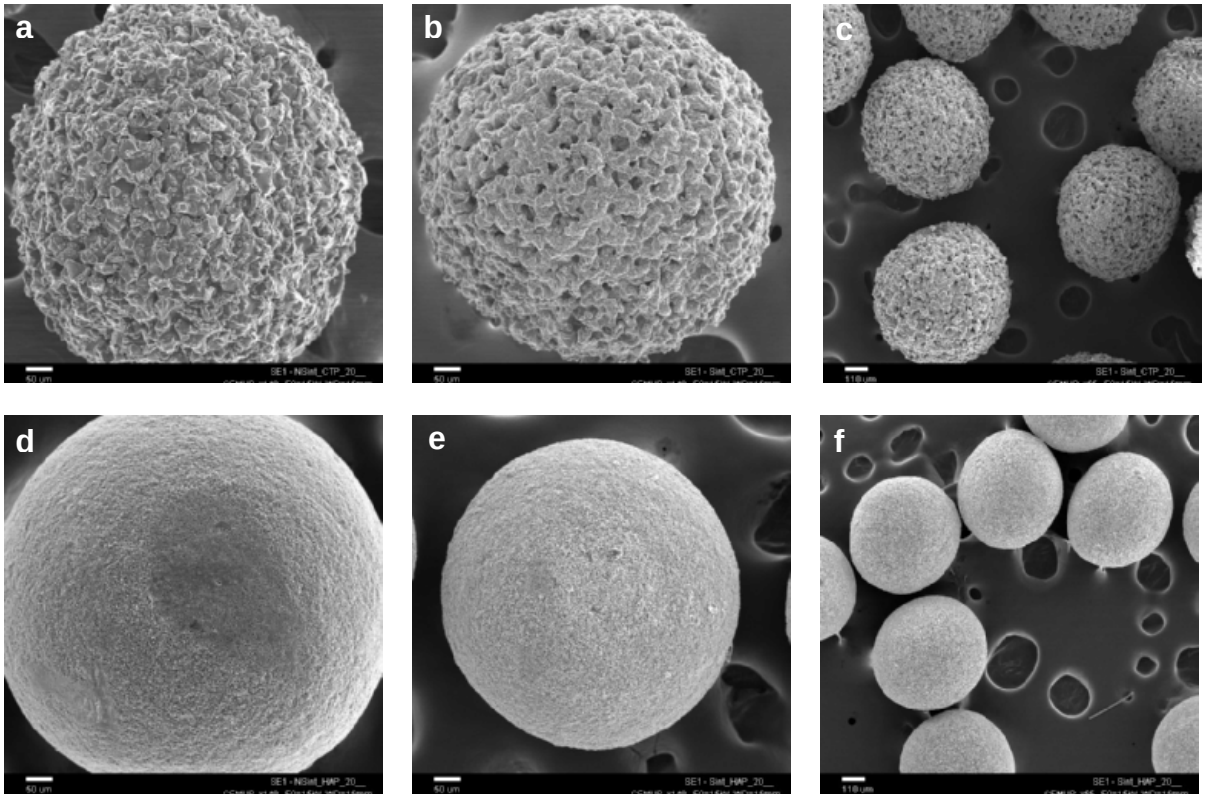

Figure 2. SEM images of CTP and HAp microspheres before and after sintering: (a) non-sintered CTP microsphere; (b, c) sintered CTP microspheres; (d) non-sintered HAp microsphere; (e, f) sintered HAp microspheres.

Non-sintered microspheres revealed, at higher magnifications, a homogeneous distribution of the ceramic phase in the alginate matrix (Fig. 3 a, d). It should be noted, however, that since the particles of HAp powder are much smaller than those of CTP, the former appear to be more densely packed. Upon sintering the polymer phase is substituted by a porous network (Fig. 3 a, b, e, f). The interconnectivity of the pores and their homogeneous distribution are clearly observed in Fig. 3b, 3c and 3f, respectively. Pores of approximately 40 μm were obtained when using CTP as the ceramic phase, while HAp resulted in pores of approximately 2 μm. Results were similar for all the formulations tested. Differences in porosity may be attributed to the different granulometries of the two compounds, which result in different packaging of the ceramic particles during drying and sintering, as already suggested.

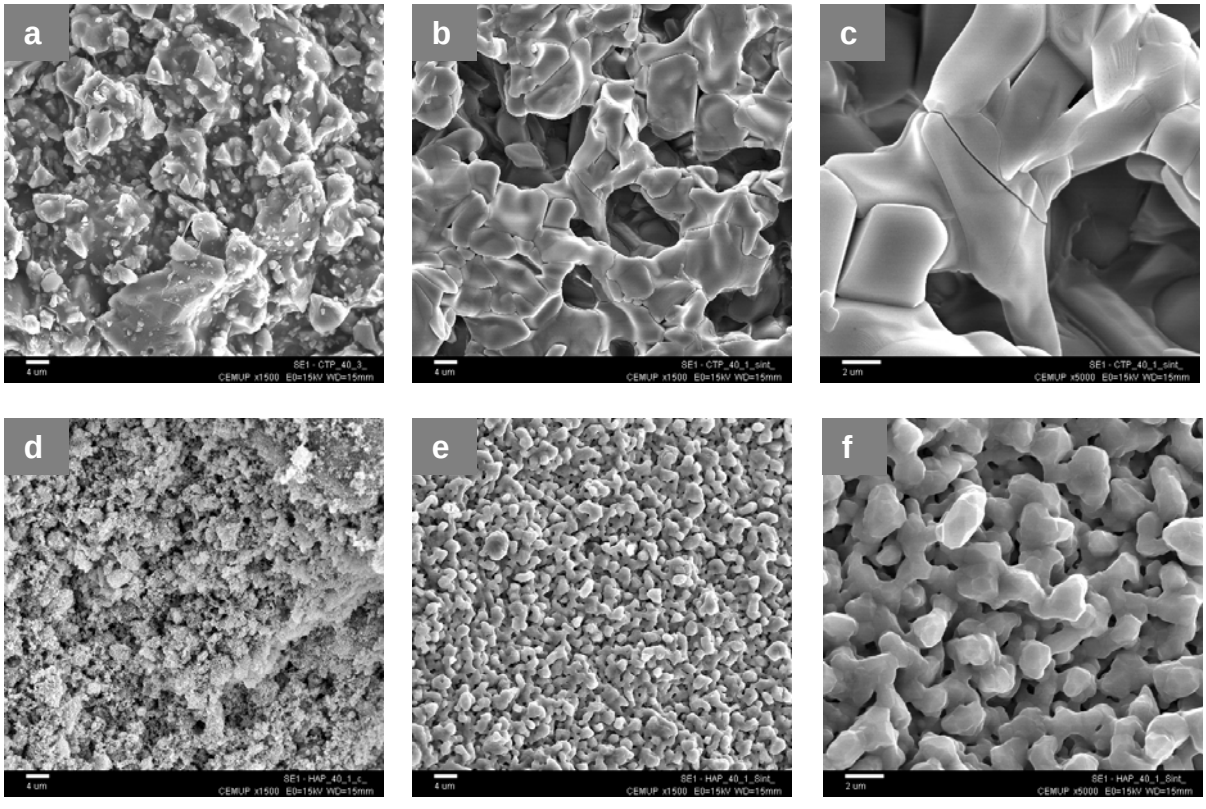

Figure 3. SEM images of the surface of CTP and HAp microspheres before and after sintering: (a) non-sintered CTP microsphere, (b, c) sintered CTP microspheres; (d) non-sintered HAp microsphere, (e, f) sintered HAp microspheres.

FTIR and XRD analysis

The FTIR spectrum of the residue powder obtained after subjecting Ca-alginate microspheres to a sintering cycle (Fig. 4) showed the presence of bands characteristic of carbonates, namely the ones in the intervals 1409-1483 cm^{-1}, 1020-1100 cm^{-1} and 800-890 cm^{-1}.[20, 21] The band at 3642 cm^{-1} is assigned to O-H vibrations, suggesting the presence of a hydroxide compound.[20, 22] XRD analysis of the residue (Fig. 5) identified it as a mixture of $Ca(OH)_2$ (portlandite) and $CaCO_3$ (calcite), confirming the results obtained by FTIR.

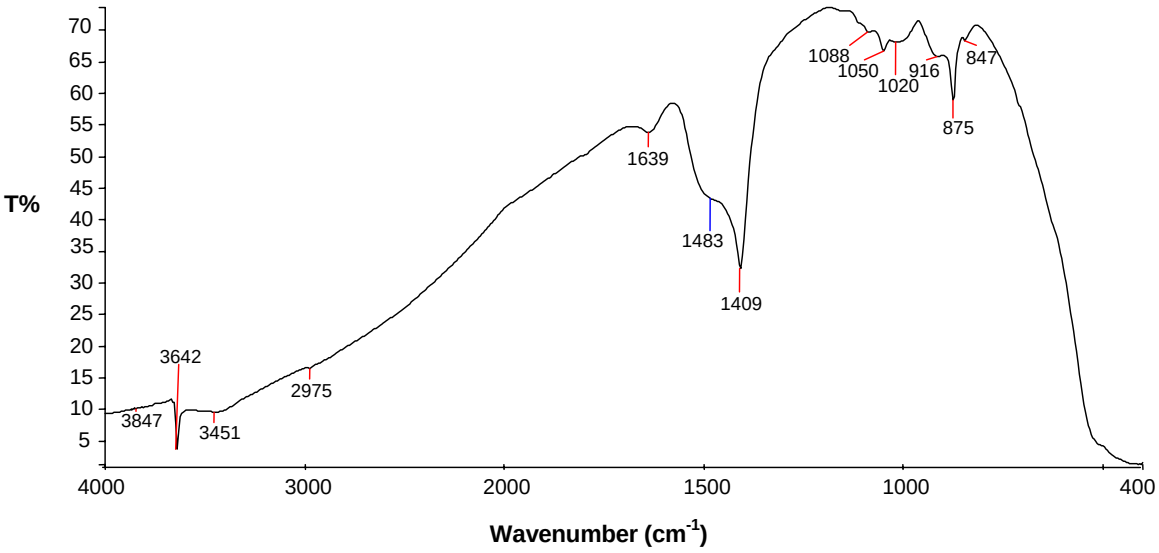

Figure 4. FTIR spectrum of the residue powder obtained after sintering Ca-alginate microspheres.

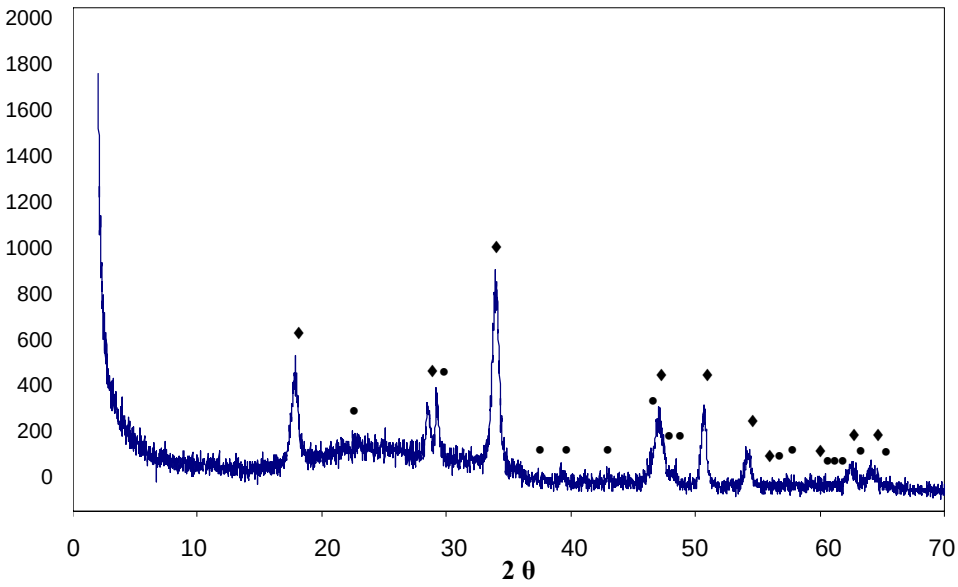

Figure 5. XRD spectrum of the Ca-alginate residue: ♦–Portlandite $Ca(OH)_2$; •–Calcite $CaCO_3$.

FTIR spectra of CTP powder and CTP microspheres are presented in Fig. 6. The changes that are observed in the spectra of CTP microspheres when compared to those of the CTP powder, suggest that a reaction between CTP and Ca-alginate must have occurred during sintering, leading to structural modifications that resulted in the appearance of new bands in the ν_3PO_4 and ν_2PO_4 phosphate zone, as well as a band at 726 cm^{-1}. These bands are more evident in the spectrum of the sample with the lowest ceramic-to-polymer solution ratio (10/3), and becomes less defined as the ceramic-to-polymer solution ratio increases, being the FTIR spectrum of the 40/3 formulation very similar to the one of CTP powder.

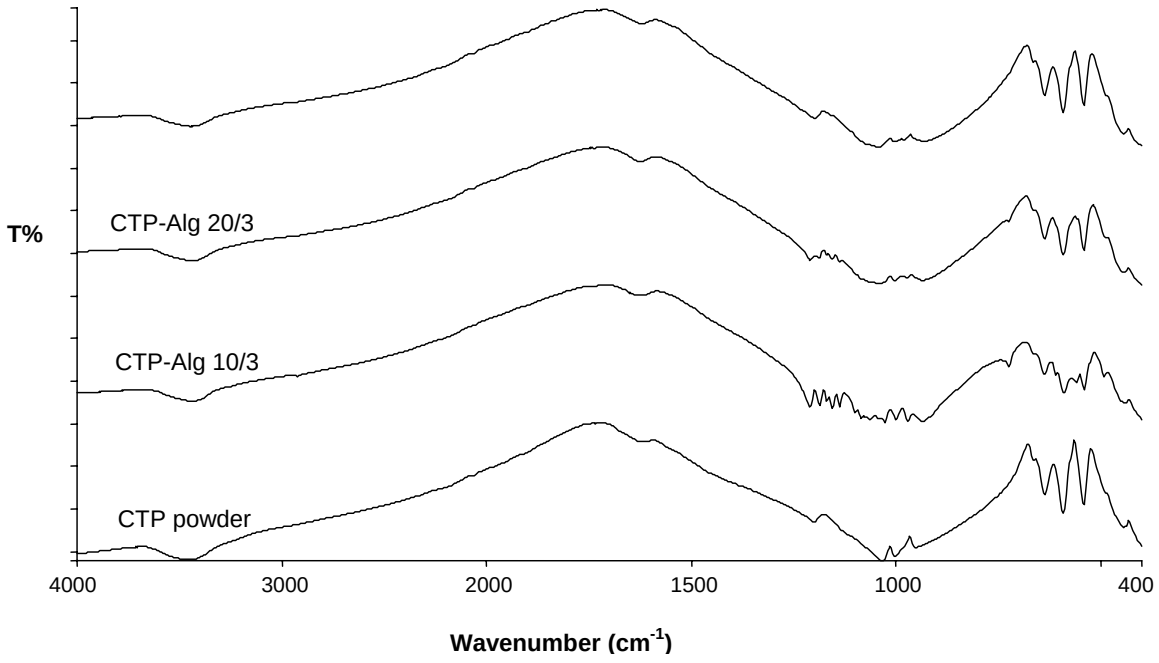

Figure 6. FTIR spectra of CTP powder and of different formulations of CTP microspheres.

Fig. 7 shows the FTIR spectrum of (a) 10/3 CTP microspheres, (b) commercial calcium pyrophosphate ($Ca_2P_2O_7$; Sigma Aldrich) and (c) titanium pyrophosphate synthesised in our laboratory. Several bands characteristic of pyrophosphate groups, namely the ones in the ranges: 900-1200 cm^{-1}, 700-770 cm^{-1} and 500-600 cm^{-1}, are common to spectra (a) and (b), suggesting the formation of a calcium pyrophosphate phase in the CTP microspheres as a consequence of the sintering process. However, the existence of that phase could not be detected by XRD analysis (Fig. 8) leading to the assumption that the phase is either amorphous or present in residual concentrations. In addition, XRD analysis showed the presence of small quantities of titanium oxide (rutile) in the sintered CTP microspheres, its

concentration being higher in the formulation 10/3. This was not unexpected considering the hypothesis described above. If a calcium pyrophosphate is formed, probably some titanium will become available to form an oxide.

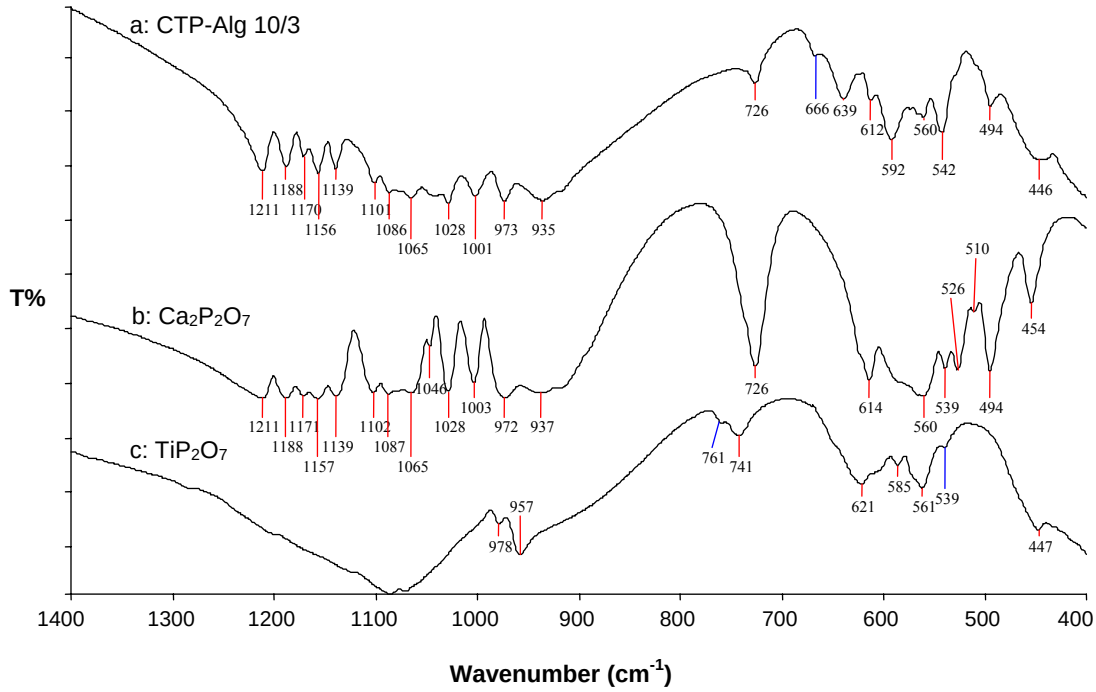

Figure 7. FTIR spectra of CTP-Alg 10/3 microspheres, calcium pyrophosphate ($Ca_2P_2O_7$), and titanium pyrophosphate (TiP_2O_7).

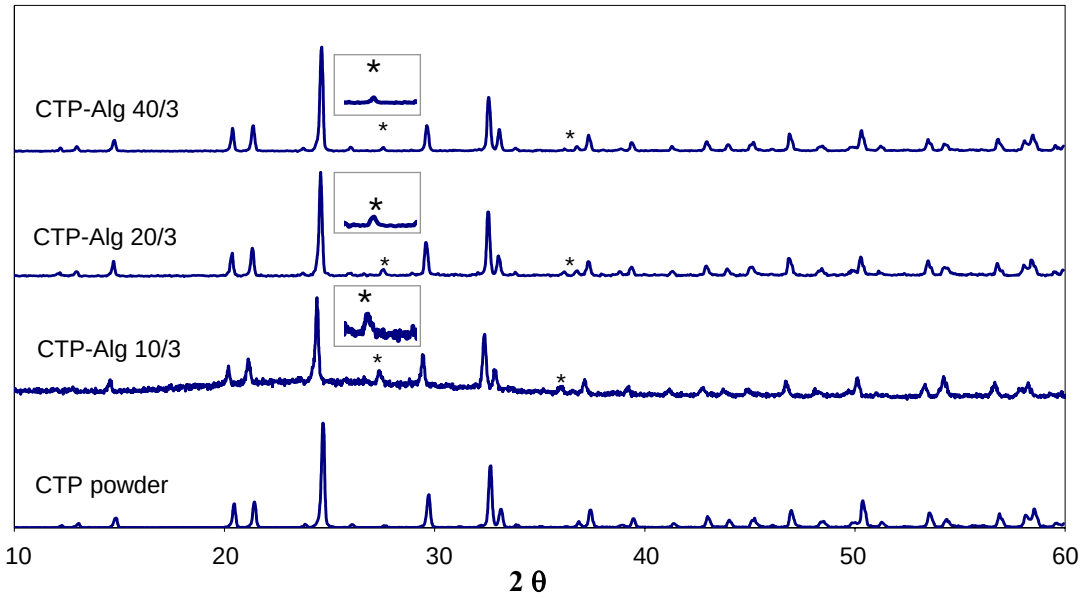

Figure 8. XRD of CTP powder and of different formulations of CTP microspheres.

In what concerns HAp microspheres, XRD analysis of the HAp powder and HAp microspheres revealed that after sintering no decomposition of the ceramic occurred, since no extraneous phases could be identified. Fig. 9 shows the FTIR spectra of HAp powder and 10/3 HAp microspheres. It is worth noting the appearance of carbonate bands at 1440 cm^{-1} and 876 cm^{-1} in the samples with the 10/3 ceramic-to-polymer solution ratio, suggesting the presence of a carbonated hydroxyapatite. This may have practical implications, since it is well established that the presence of carbonates in calcium phosphate materials is an important factor contributing to the *in vivo* integration of the implant. For the 20/3 and 40/3 formulations, the FTIR spectra of the microspheres are practically identical to the one of HAp, but the presence of carbonate bands is less evident. No signs of calcium hydroxide were observed in the sintered CTP-alginate and HAp-alginate microspheres.

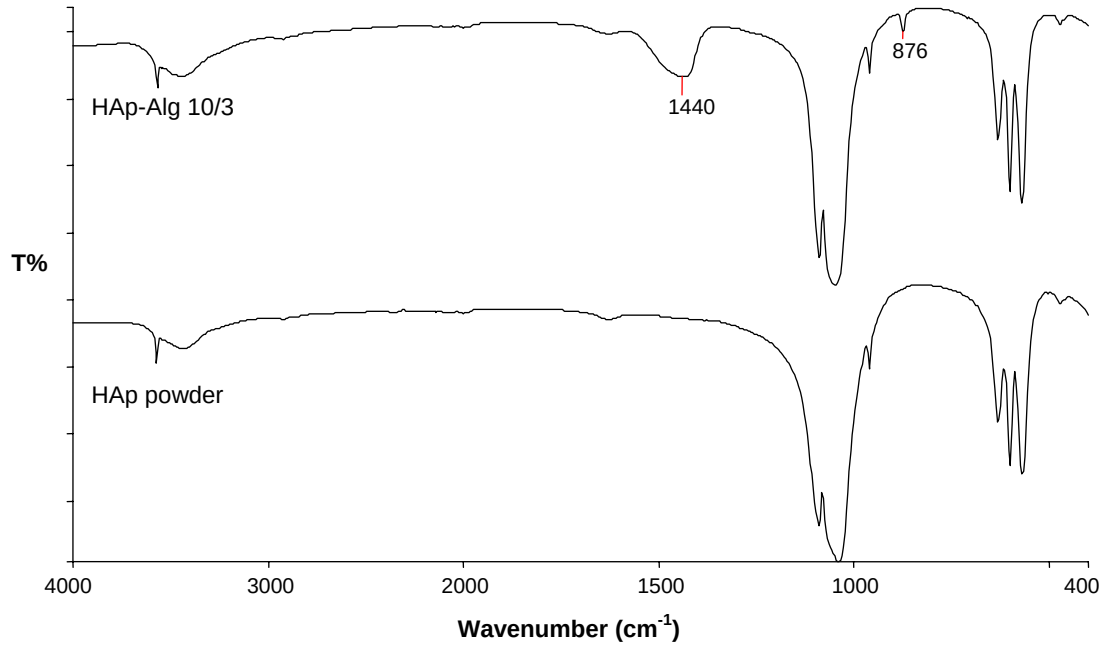

Figure 9. FTIR spectra of HAp powder and HAp-Alginate 10/3 microspheres.

In order to clarify if a carbonated apatite has been obtained, or if the carbonate bands were due to formation of a calcium carbonate residue, resultant from the burning of alginate, 10/3 HAp microspheres were subjected to a second sintering cycle at 1100ºC for 1 h. When heated to this high temperature, any free calcium carbonate would disappear since calcium carbonate (calcite) decomposes at approximately 825ºC into CaO and CO_2.[23] The FTIR

spectra of the re-heated HAp microspheres still presented the carbonate bands, confirming the presence of a carbonated apatite.

DISCUSSION

There are several reports described in the literature concerning the development of HAp or HAp-polymer granulates with spherical geometry.[8-11, 24-28] Most of the methods used to produce microspheres have the disadvantage of using organic solvents or oils thus needing several washing stages to eliminate them. For instance, Sunny *et al*[10] produced HAp/chitosan microspheres by the dispersion of HAp/chitosan slurry in liquid parafin, with the addition of gluteraldehyde to harden the spheres. Sivakumar *et al*[11, 28] developed a method to produce coralline HAp/chitosan and coralline HAp/gelatin composite microspheres using a PMMA dispersion solution containing toluene as a crosslinking agent. The experimental method proposed in this study has the advantage of not using such reactants, thus allowing the recovery of the microspheres without the need of fastidious washing processes.

Another advantage of the present methodology is the possibility of producing spherically shaped particles with uniform sizes. Irregularly or densely packed granules have been described in the literature as being the cause of inflammatory reactions and slower bone formation.[29] Uniformly packed spherical particles would lead to a regular inter-particle porosity, thus contributing to easier migration of bone cells. Moreover, since our aim is to use the microspheres in an injectable form, their flowing properties during injection will be more predictable if their shape and size are more regular.

Since these microspheres may also be used to deliver growth factors and other proteins, their porosity is a critical parameter since it may strongly influence their loading capacity. The raise in porosity that occurs upon sintering will significantly increase the surface area and allow higher loading yields.

This investigation has shown that the ratio between the ceramic phase and the polymer solution is of critical importance in the composition of the sintered microspheres. In the case of the CTP-alginate microspheres, a calcium pyrophosphate was formed after sintering, and its concentration increases as the ceramic to polymer ratio decreases. When subjected to the same heating cycle used for sintering the microspheres, CTP powder does not suffer any

change in composition. These observations suggest that the formation of the pyrophosphate phase is related to the presence of alginate. Sodium alginate forms a relatively stable hydrogel of calcium alginate through ionotropic gelation in the presence of Ca^{2+}. A possible mechanism to explain the formation of the pyrophosphate phase after sintering is the interaction between calcium ions from the alginate and phosphate groups from the ceramic, possibly forming an acid salt. When a solid orthophosphate, which contains hydrogen bonds (e.g. $CaHPO_4$) is heated to an appropriate temperature, there is a loss of water accompanied by pyrophosphate formation.[30, 31] The amount of pyrophosphate formed is related to the number of hydrogen bonds present in the system. Pyrophosphate ion is a well-known inhibitor of apatite formation.[31-33] Legeros *et al*.[33] have demonstrated that the presence of pyrophosphate ions are efficient in promoting the formation of amorphous calcium phosphates. The microspheres prepared in this investigation are aimed at promoting mineralization. Therefore, a formulation with a high ceramic to polymer ratio will be more adequate.

With HAp-alginate microspheres it was observed that the sintering process leads to the formation of carbonated hydroxyapatite. The carbonate ions were probably incorporated in the HAp structure according to the following reaction: [34]

$$2OH^-\,(\text{apatite}) + CO_2 \rightarrow CO_3^{2-}\,(\text{apatite}) + H_2O$$

which was probably favoured by an increase of the local concentration of CO_2 resulting from the burning of alginate. This is in agreement with the fact that carbonate bands are more intense in the low ceramic to polymer ratio formulations. The substitution of carbonate in well and poorly crystallized hydroxyapatite is well documented in the literature. It has been suggested that planar CO_3 ion substitutes for tetrahedral PO_4 groups in hydroxyapatite prepared at room (or body) temperature while in high temperature preparations (about 1000ºC), CO_3 substitutes for OH.[31]

CONCLUSIONS

Porous ceramic microspheres with a uniform size were prepared using a novel methodology consisting of sintering spherical particles, obtained by droplet extruding a mixture of ceramic powders and sodium alginate followed by ionotropic gelation of the polymeric matrix in the presence of Ca^{2+}. Possibly, microspheres with different porosities may be produced by this method, by varying the granulometry of the ceramic powders. The ratio between the ceramic phases and the polymer solution is a critical parameter in the composition of the sintered microspheres and must be carefully selected. These materials could find applicability in the field of bone regeneration, both as injectable bone-filling materials and drug delivery matrices.

Acknowledgments

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Chapter VI

Adhesion and proliferation of human osteoblastic cells seeded on injectable hydroxyapatite microspheres*

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ABSTRACT

In the present work, the interaction of human osteoblast-like MG63 cells with novel hydroxyapatite (HAp) microspheres was investigated. Cells were seeded on the microspheres and incubated for up to 7 days. The cell-material constructs were visualised by scanning electron microscopy and confocal laser scanning microscopy, and the rate of cell proliferation was estimated using the Neutral Red assay. The results showed that osteoblastic cells were able to adhere and proliferate on the HAp microspheres, which are intended to be used as injectable support-materials for bone regeneration.

Keywords: Injectable microspheres, hydroxyapatite, bone regeneration, osteoblast-like MG63 cells

INTRODUCTION

The use of injectable biomaterials for bone regeneration purposes has received much attention recently.[1] The main advantage of such strategy relies on the possibility of filling

defects of different shapes and sizes, while requiring only minimally invasive techniques for implantation, which provide less patient discomfort. Microparticles, suspended on appropriate vehicles, are among the most common forms of injectable materials. Once implanted, they are expected to conform to the irregular implant site, with more or less close packing, and to encourage host cell migration, attachment, proliferation and differentiation. The interstices between the particles, if presenting an appropriate size, may also provide a space for both tissue and vascular ingrowth.

In a previous work, a novel route for the preparation of calcium-phosphate microspheres was described.[2,3] The ceramic granules are first mixed with alginate, which enables the preparation of homogeneous spherical particles through ionotropic gelation in the presence of Ca^{2+}. The spherical particles are subsequently sintered to burn-off the polymer and fuse the ceramic granules, producing pure hydroxyapatite (HAp) microspheres.[2,3] In the present study, the ability of HAp microspheres to support the adhesion and growth of human osteoblastic cells was investigated using the MG63 cell line.

MATERIALS AND METHODS

HAp microspheres were prepared as previously described.[2,3] Briefly, HAp powder (CAM Implants) pre-heated at 1000ºC was mixed with sodium alginate solution (3% w/v) at a ratio of 0.2 w/w and well homogenised. The paste was extruded drop-wise into a 0.1M $CaCl_2$ crosslinking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min. The size of the microspheres was controlled by regulating the extrusion flow rate using a syringe pump and by applying a coaxial air stream (Encapsulation Unit Var J1; Nisco). At completion of the gelling period, microspheres were recovered and rinsed in water in order to remove the excess $CaCl_2$. Finally, they were dried overnight in a vacuum-oven at 30ºC, and then sintered at 1100ºC for 1 h, with a uniform heating rate of 5ºC/min from room temperature.

The size of the microspheres was determined using a laser scanner particle size analyser (Coulter Electronics), and their morphology was analysed by optical microscopy.

Cell culture studies were performed using MG63 osteoblast-like cells, which express a number of features characteristic of relatively immature osteoblasts. Cells were routinely

maintained in α-minimal essential medium (α-MEM) supplemented with 10% v/v foetal calf serum, 2.5 μg/mL fungizone and 50 μg/mL gentamicine. Microspheres were steam sterilised (120ºC, 20 min) and pre-incubated in culture medium overnight in non-tissue culture plates, to avoid cell adhesion on the bottom of the wells. MG63 cells were seeded on the microspheres at 500 cells/mg and incubated at 37ºC in a humidified atmosphere of 5% v/v CO_2 in air for 4 h, or 1, 3, 5 and 7 days with the medium being replaced every 2 days. Cell proliferation was estimated using the neutral red (NR) assay. Cells seeded on tissue culture grade polystyrene (TCPS) plates were used as a control. Cell morphology was visualised at different time points by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

RESULTS AND DISCUSSION

The granulometric analysis of the microspheres (Fig. 1a) revealed that the size distribution of the main population of particles is narrow and follows a normal distribution, with 90% (in volume) of the particles being smaller than 600 μm and having an average diameter around 550 μm. A second population of microspheres with an average diameter around 1000 μm and corresponding to approximately 4% of the particles was also identified. Sieving could easily eliminate these larger microspheres, which result from occasional particle-particle aggregation during the extrusion process. An optical micrograph of the microspheres is presented in Fig. 1b and illustrates their uniformity.

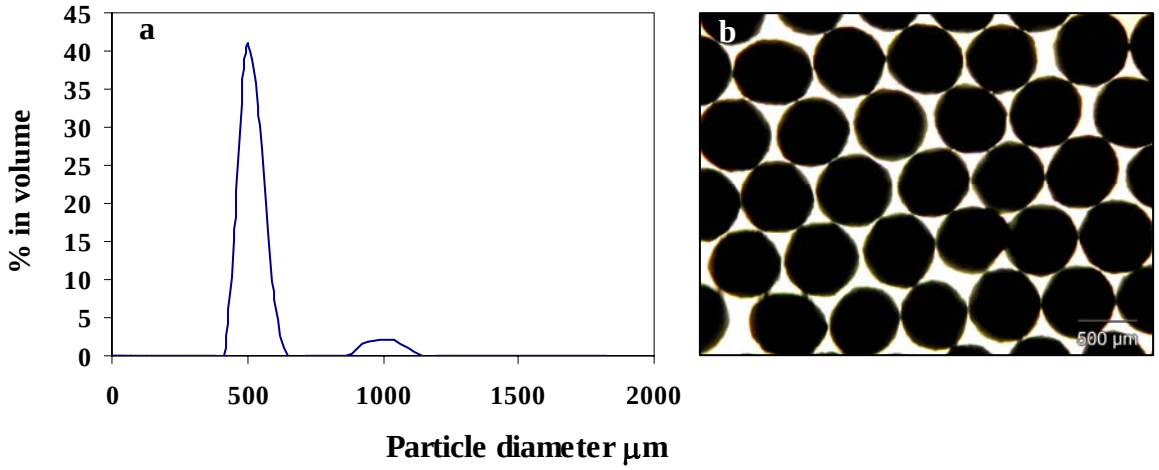

Figure 1. (a) Particle size distribution; and (b) optical microscopy image of the HAp microspheres.

SEM analysis of the cell-microspheres constructs revealed that after an initial period of 4 h (Fig 2a), some adherent cells could already be observed on the surface of the microspheres. Because the ability of cells to attach, adhere and spread will influence their capacity to proliferate and differentiate in contact with the material, this initial phase is of critical importance.[4] After 1 day (Fig. 2b and 2c) several cells exhibiting a spindle-like morphology were dispersed on the surface of the microspheres. Some round cells (possibly mitotic cells) were also present.

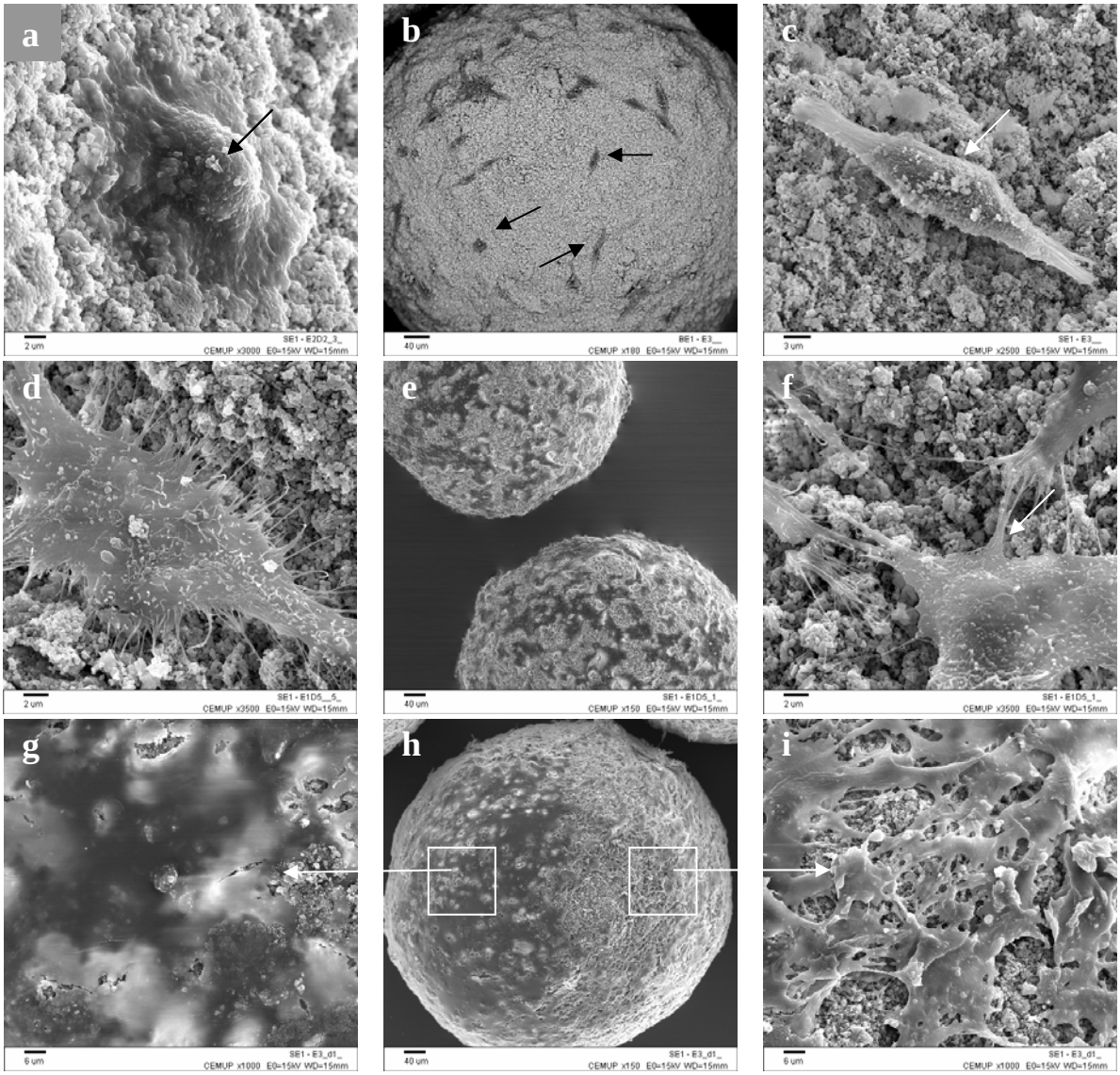

Figure 3. SEM images (several magnifications) showing MG63 human osteoblastic cells on the surface of hydroxyapatite microspheres after an incubation period of: (a) 4 h; (b and c) 1 day (image 3b was obtained with the backscattered electrons mode); (d-f) 5 days; and (g-i) 7 days.

At day 5 (Fig. 2d to 2f), cells were well flattened exhibiting numerous filopodial-like extensions and cell-cell contact points. Finally, at day 7 (Fig 2g to 2i) the microspheres were almost completely covered by cells that formed continuous layers in some regions.

The distribution of cells on HAp microspheres (day 5) was also visualised using CLSM. (Fig. 3a). Pictures constructed by superimposing images obtained using both the fluorescence and reflection channels showed numerous cells on the surface, a result similar to the one obtained by SEM.

The number of viable cells on the surface of the microspheres was evaluated by performing the NR assay at different time points. This assay is based on the incorporation of a vital colorant by viable cells, and its subsequent fixation at anionic sites on the surface of lysosomal membranes. As any alteration of the membranes will result in a diminished fixation of the colorant, only viable cells will be stained, and so the intensity of the developed coloration allows the indirect quantification of the number of viable cells in the sample. Fig. 3b shows that the number of viable cells, both on the surface of the microspheres and on control TCPS plates, gradually increased along the 7 days in culture, indicating that cells were actively proliferating.

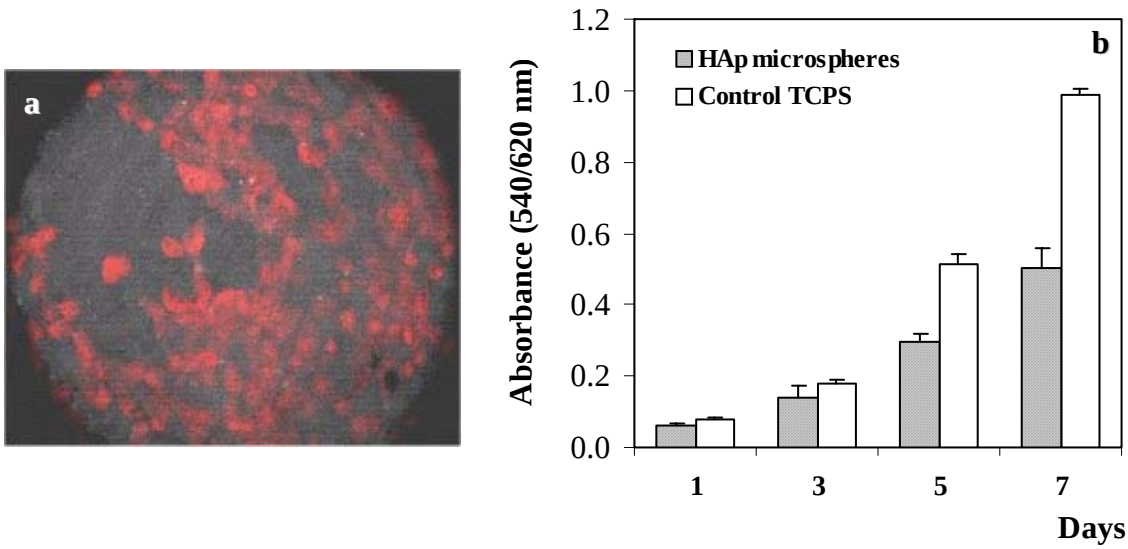

Figure 2. (a) CLSM image showing osteoblastic cells (red areas) on the surface of HAp microspheres (day 5); and (b) proliferation of cells on HAp microspheres estimated by the NR assay.

CONCLUSIONS

In this study it was demonstrated that HAp microspheres are able to support human osteoblastic cells adhesion and proliferation. Further studies to evaluate the influence of these materials on the expression of the osteoblastic phenotype are in progress.

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Chapter VII

Proliferation, activity and osteogenic differentiation of bone marrow stromal cells cultured on calcium titanium phosphate microspheres*

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ABSTRACT

In this study, the behaviour of bone marrow stromal cells cultured on calcium titanium phosphate (CTP) microspheres was analysed. Cell adhesion and proliferation were estimated by the neutral red assay and by total DNA quantification. Morphology and deposition of extracellular matrix were assessed by confocal laser scanning microscopy and/or scanning electron microscopy. The expression of the osteoblastic phenotype was evaluated by monitoring alkaline phosphatase activity and osteocalcin secretion. Results revealed that cells were able to attach and spread on the surface of CTP microspheres, and gradually grow into nearly confluent monolayers. Moreover, cells were able to bridge adjacent microspheres forming microsphere-cell clusters. Cells produced an abundant amount of fibrillar extracellular matrix that covered the substrate surface. Alkaline phosphatase activity peaked around days 7-14 and then decreased until day 21. Cells secreted osteocalcin, with higher levels being detected at day 14 than at day 21.

Taken together these results suggest that CTP microspheres are appropriate scaffolds for the

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growth and differentiation of cells along the osteoblastic lineage.

Keywords: Calcium titanium phosphate, microspheres, bone marrow stromal cells, osteogenic differentiation

INTRODUCTION

Microparticulate systems offer exciting possibilities across many fields of regenerative medicine and have been extensively studied for several applications. The most important ones include delivery vehicles for drugs, proteins or genes and matrices for imunoisolation of transplanted cells. Recently, attention has been drawn to the use of microparticles as injectable scaffolds for tissue regeneration.[1-5] Microparticles can be seeded with autologous cells prior to implantation, functioning as cell-carriers, or designed to encourage host cell migration, attachment, proliferation and differentiation once implanted. The main advantage of this approach, compared to the traditional block scaffolds, is that small particles can be combined with a vehicle and be administered by injection, thus giving the possibility of filling defects of different shapes and sizes though minimally invasive surgery. Upon implantation, the microspheres-vehicle system is expected to easily conform to the irregular implant site, whereas the interstices between the particles may provide a space for both tissue and vascular ingrowth, as required for effective healing.

In cell culture technology, microparticles have been used as an alternative culture-substrate to *in vitro* growth of anchorage-dependent cells, for large-scale cell expansion and/or production of numerous cell products including vaccines, enzymes, antibodies, etc. The concept of culturing cells as monolayers on the surface of small spheres, commonly referred as microcarriers, was first conceived by Van Wezel[6] and different types of microcarriers are currently available in the market. The large surface area-to-volume ratio provided by microcarriers allows easy propagation and high cell yields, while requiring much less culture medium and space than traditional monolayer culture techniques.

For the culture of some specific types of cells, a microcarrier culture may also present other advantages. For example, the phenotype of chondrocytes[7,8] is better retained in microcarriers than in flat surfaces. Although the exact reasons for this remain uncertain, it has been suggested that the thee-dimensional structure provided by microcarriers may probably better

mimic the environment found *in vivo*.[7]

In a previous work, a methodology for the preparation of calcium phosphate microspheres has been described.[9,10] Ceramic granules of hydroxyapatite (HAp) or calcium titanium phosphate (CTP) were mixed with sodium alginate, and microspheres were prepared by drop formation under coaxial air flow, followed by ionotropic gelation in the presence of Ca^{2+}. The particles were subsequently sintered to burn-off the polymer and aggregate the ceramic granules. Besides its simplicity, one of the most important advantages of this method is that it allows the preparation of spherical-shaped particles with an adequate and uniform size.

Calcium phosphate materials have long been recognised as adequate scaffolds for bone regeneration and some are already used in clinical practice. Moreover, they are amenable to sterilization, which is advantageous for both *in vitro* and *in vivo* applications. We have already reported that HAp microspheres promote the attachment and proliferation of human osteoblastic MG63 cells.[5] In the present work, the behaviour of bone marrow stromal cells, recognized as osteogenic cells, cultured on CTP microspheres was investigated though analysis of cell attachment and morphology, cell proliferation, deposition of extracellular matrix and expression of osteogenic differentiation markers.

MATERIALS AND METHODS

Preparation and characterisation of microspheres

Calcium titanium phosphate [CTP, $CaTi_4(PO_4)_6$] microspheres were prepared as reported previously.[9,10] Briefly, CTP was synthesised by solid-state reaction as described elsewhere[10] and mixed at a ratio of 0.4 with 2% w/v sodium alginate (Pronova Biopolymers) aqueous solution. After homogenisation the paste was extruded drop-wise into a 0.1 M $CaCl_2$ crosslinking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min. The size of the microspheres was controlled by regulating the extrusion flow rate using a syringe pump and by applying a coaxial air stream (Encapsulation Unit Var J1, Nisco). At completion of the gelling period, microspheres were recovered and rinsed in water in order to remove the excess $CaCl_2$. Finally, they were dried overnight in a vacuum-oven at 30ºC, and then sintered at 1100ºC for 1 h, with a uniform heating rate of

5ºC/min from room temperature.

Physicochemical characterisation was carried out by Fourier transform infrared spectroscopy (FTIR). For analysis, microspheres were reduced to powder and analysed as KBr pellets using a Perkin Elmer System 2000 spectrometer. The size of the microspheres was estimated using an inverted platen microscope (Olympus PME3-ADL) equipped with an ocular micrometer with an accuracy of 10 μm, and their morphology was analysed by digital imaging and scanning electron microscopy (SEM). Tissue culture treated polystyrene (TCPS) microcarriers (Biosilon, Nunc™) were used as a control and were also characterised in terms of size and by SEM.

Cell isolation and culture

Rat bone marrow stromal cells were used under consent of the local ethic committee, and were isolated and cultured according to the method of Maniatopoulos *et al*.[11] Briefly, the femurs and tibias of Wistar rats (male, 4 weeks old) were aseptically excised from the hind limbs, the epiphyses were cut off and the medullary space was flushed with α-minimal essential medium (Gibco) supplemented with 10% v/v foetal bovine serum, 50 μg/mL gentamicin and 0.3 μg/mL fungizone (standard medium). Cells were plated in 75-cm^2 flasks and incubated at 37ºC in a humidified atmosphere of 5% v/v CO_2. Non-adherent cells were removed after 24 h. Adherent cells were subsequently cultured for 1 week, with the medium renewed every 3 days. Cells from the first passage were used.

Cell seeding on microspheres

Microspheres were steam sterilised (120ºC, 20 min), placed in non-treated 96-well plates (14 mg/well, ca. 1-cm^2 surface area) to avoid cell adhesion to the bottom of the wells, and pre-incubated in standard medium overnight. TCPS microcarriers (4.5 mg/well, ca. 1-cm^2 surface area) were used as a control. Cells were seeded at 1.2×10^4 cells/cm^2 in half of the total volume to be used, and cell-microspheres constructs (n=5) were incubated (37ºC, 5% v/v CO_2 in air) in standard medium supplemented with 10^{-8} M dexamethasone, 50 μg/mL ascorbic acid and

10 mM β-glycerophosphate. The remaining medium was added after 24 h. Medium was renewed every 3 days. Materials without cells processed and cultured under the same conditions were used as references.

Cell adhesion and morphology

The number of adherent cells on the microspheres was assessed by the neutral red (NR). Cell distribution and morphology was analysed by SEM and by confocal laser scanning microscopy (CLSM). All assays are described in detail below.

NR assay

The culture medium was discarded and replaced by fresh medium containing 50 µg/mL NR (Sigma). Plates were incubated at 37ºC for 3 h. At the end, the supernatant was discarded and microsphere-cell constructs washed twice with phosphate-buffered saline (PBS). The NR absorbed by the cells was extracted with 1% v/v acetic acid in 50% v/v aqueous ethanol. After being well homogenised on a shaker platform, the supernatants were transferred to another 96-well plate, and the optical density was read at 540 nm with reference to 620 nm ($OD_{540/620}$) in a microplate spectrophotometer. Cell number was determined using the linear region of a standard curve where absorbance was plotted against known numbers of cells counted using a Neubauer chamber.

SEM

Microsphere-cell constructs were washed twice with PBS and fixed in 1.5% v/v glutaraldehyde in 0.14 M sodium cacodylate (pH 7.4) for 30 min at room temperature. Dehydration was carried out by sequential immersion in serial diluted ethanol solutions of 50, 60, 70, 80, 90 and 100% v/v. Samples were then transferred to hexamethyldisilazane (HMDS) and air-dried at room temperature overnight. Finally, the microsphere-cell constructs were sputter-coated with gold using a JEOL JFC-100 Fine Coat Ion Sputter device, and observed using a JEOL JSM-6301F scanning microscope.

CLSM

Microsphere-cell constructs were washed twice with PBS, fixed in 4% v/v formaldehyde (methanol-free; Polyscience) for 15 min, permeabilized with 0.1% v/v Triton X-100 for 5 min and incubated in 10 mg/mL bovine serum albumin and 100 µg/mL RNAse for 45 min at room temperature. F-actin filaments were stained with Alexafluor-conjugated phalloidin (Molecular Probes) for 20 min and nuclei were counterstained with 10 µg/mL propidium iodide (Sigma) for 10 min. Finally, samples were washed with PBS and mounted in Vectashield®. CLSM images were acquired on a BioRad MRC 600 microscope.

Cell proliferation and differentiation

Cell proliferation was assessed by the NR assay (described above) and by total DNA quantification. Levels of alkaline phosphatase (ALP) activity and osteocalcin (OC) secretion, markers of osteoblastic differentiation, were determined.
Microsphere-cell constructs were rinsed twice with PBS and cell lysates were obtained by brief sonication in 1% v/v Triton X-100. The homogenates were centrifuged to remove ceramic particles and used in ALP and total DNA assays. The supernatant culture media were reserved for OC analysis. All assays are described in detail below.

Total DNA quantification

DNA content was determined by the method of Labarca and Paigen[12] using calf thymus DNA as a standard. Briefly, cell lysates were mixed with 0.1 µg/mL bisbenzimide (Hoechst 33258; Sigma) in 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2 M NaCl (pH 7.4) and fluorescence was read with excitation at 356 nm and emission at 458 nm.

ALP colorimetric analysis and histochemical staining

ALP was assayed as the hydrolysis of the artificial substrate *p*-nitrophenol phosphate. Cell lysates were incubated in 2 mM *p*-nitrophenol phosphate, 0.2 M bicarbonate buffer (pH 10),

0.05% v/v Triton X-100, and 4 mM $MgCl_2$ for 60 min at 37°C. The reaction was stopped by adding 1 M NaOH, and the product was quantified at 405 nm, using a set of *p*-nitrophenol standards.

For histochemical staining, microsphere-cell constructs were washed twice with PBS, fixed in 4% v/v formaldehyde for 15 min, washed in water and incubated for 30 min in Naphtol AS-MX phosphate/ Fast Violet B salt (Sigma) at room temperature and in the dark. Finally, cells were washed in water, air dried, and observed under a stereo microscope (Olympus SZX9).

OC quantification

For analysis of OC, supernatant media were centrifuged (2 min at 10,000 rpm) to remove cell debris, stored at –20ºC and then thawed before analysis. OC was assayed using the Rat-MID Osteocalcin ELISA kit (Osteometer BioTech A/S, Denmark) according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± standard deviation (n=5) and were analysed using the Mann-Whitney *U* test. Differences between groups were considered statistically different when $p<0.05$.

RESULTS

Preparation and characterisation of CTP microspheres

The CTP-alginate suspension dropped into $CaCl_2$ solution formed gel beads instantaneously. During sintering, the polymer was burned off and the CTP granules became associated, while the original spherical-shape of the particles was maintained. The average diameter of CTP microspheres was 607±30 μm (n=20), as assessed by optical microscopy. TCPS microcarriers have an average diameter of 205±120 μm. Fig.1 illustrates the spherical-shape of CTP

particles (Fig. 1a) as well as their uniform size (Fig. 1b). CTP microspheres present a rougher surface than TCPS microcarriers (Fig. 1c).

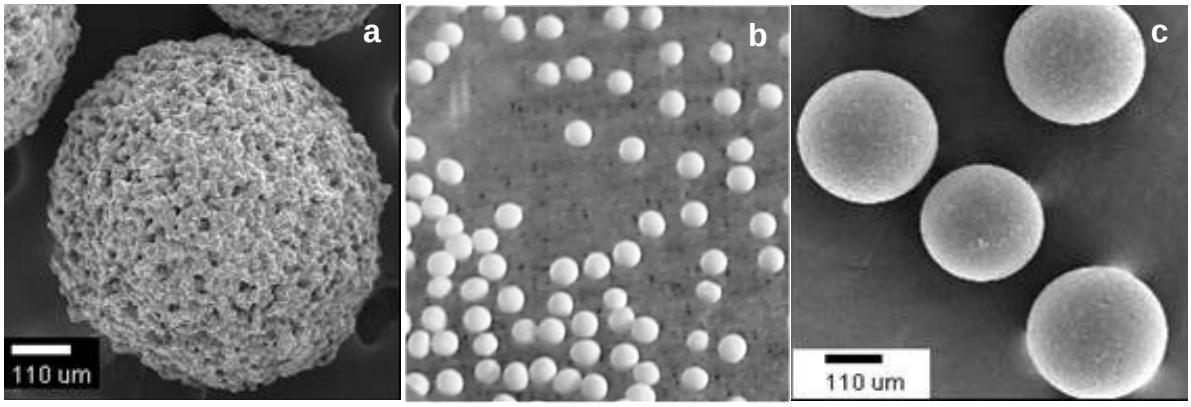

Figure 1. (a) SEM micrograph and (b) photograph (digital camera) of CTP microspheres illustrating their spherical shape and uniform size; (c) SEM micrograph of TCPS microcarriers.

Physicochemical characterisation was performed by FTIR. The FTIR spectra of CTP powder and CTP microspheres were identical, showing that the ceramic kept its integrity, and for that reason were not included here. In a previous work[10] it was demonstrated that depending on the ceramic-to-polymer ratio used in the preparation of the microspheres, the structure of CTP may become altered during the subsequent sintering process. In the present study a high ceramic-to-polymer ratio was selected to guarantee the absence of extraneous phases, namely of calcium pyrophosphates, in the sintered microspheres. Further details on the characterisation of CTP microspheres are provided elsewhere.[9,10]

Culture of bone marrow stromal cells on CTP microspheres

Cell attachment, adhesion and spreading

Cells were seeded at approximately the same density per surface area on both materials (1.2×10^4 cells/cm^2). The number of adherent cells after 24 h of incubation was estimated

using the NR assay (Fig. 2a). Approximately 16% of the seeded cells attached to CTP microspheres, whereas 46% attached to TCPS microcarriers. After 24 h of culture, both spindle-shaped and well-flattened cells were observed by SEM (Fig. 2b) on the surface of CTP microspheres.

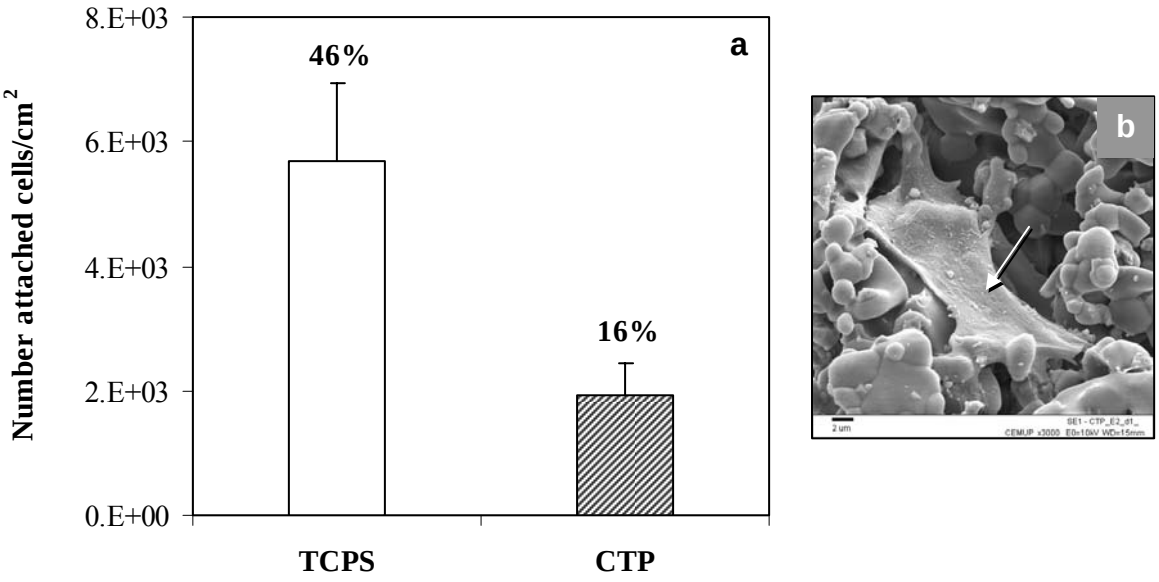

Figure 2. (a) Number of cells per cm^2 attached to CTP and TCPS microspheres following 24 h of incubation. Data labels represent the percentage of attached cells in relation to the amount of cells initially employed (1.2×10^4 cells/cm^2); (b) SEM image of a flattened cell on the surface of a CTP-microsphere at 24 h.

CLSM images of bone marrow stromal cells after 5 days of culture on CTP microspheres are presented in Fig. 3. Cells were found to be reasonably well distributed between microspheres (Fig. 3a). Cells were able to spread throughout the substrate surface and adopt a typical osteoblast-like morphology (Fig. 3b). Numerous cell-cell contact points were detected and, in some regions, cells started to form continuous cell layers. At higher magnification (Fig. 3c), filamentous actin organised in thick stress fibbers could be visualised.

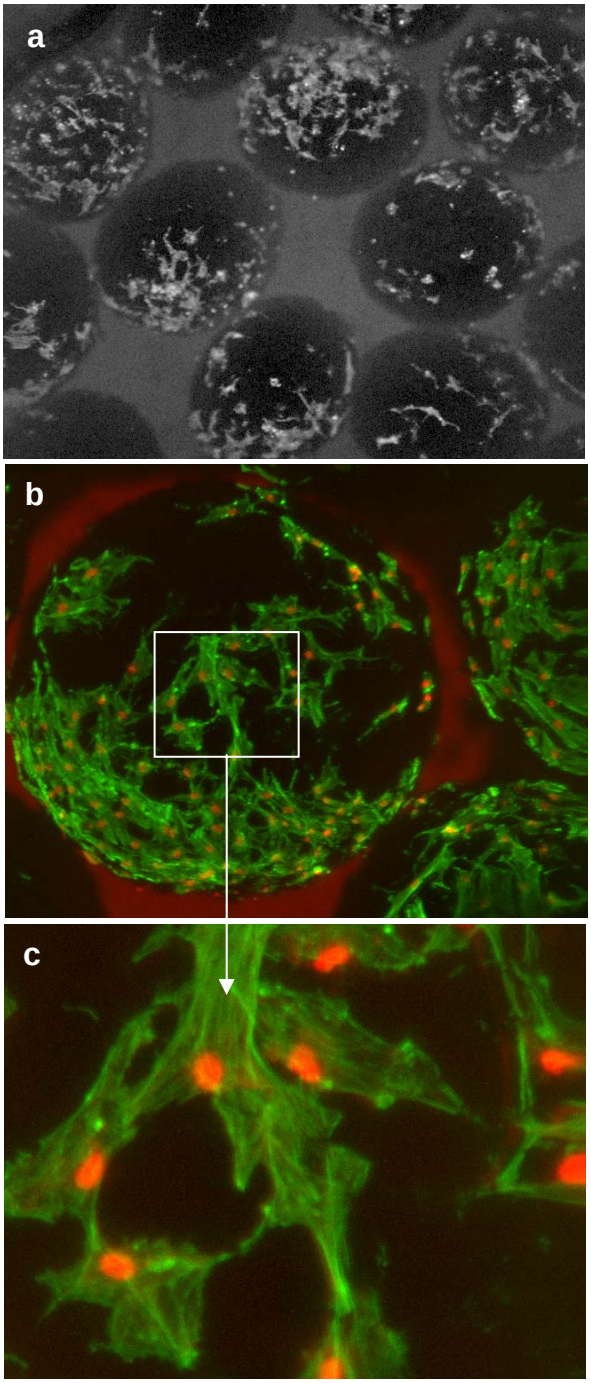

Figure 3. CLSM images of bone marrow stromal cells cultured for 5 days on the surface of CTP microspheres, showing: (a) a uniform distribution of cells between microspheres (original magnification, 50×); (b) cells with a typical osteoblast-like morphology throughout the surface (original magnification, 100×); and (c) filamentous actin organised in thick stress fibbers (original magnification, 800×). Cells were stained with phalloidin (F-actin) and counterstained with propidium iodide (DNA).

Cell proliferation and production of extracellular matrix (ECM)

Cell proliferation, estimated by the NR assay and by total DNA quantification, is depicted in Fig. 4a and 4b. Cells proliferated during the first 2 weeks of culture as demonstrated by both assays. The number of cells increased approximately 12-fold in relation to day 1, both on CTP microspheres and on TCPS microcarriers. The gradual increase in DNA content during the same period followed a similar pattern (Fig. 4b).

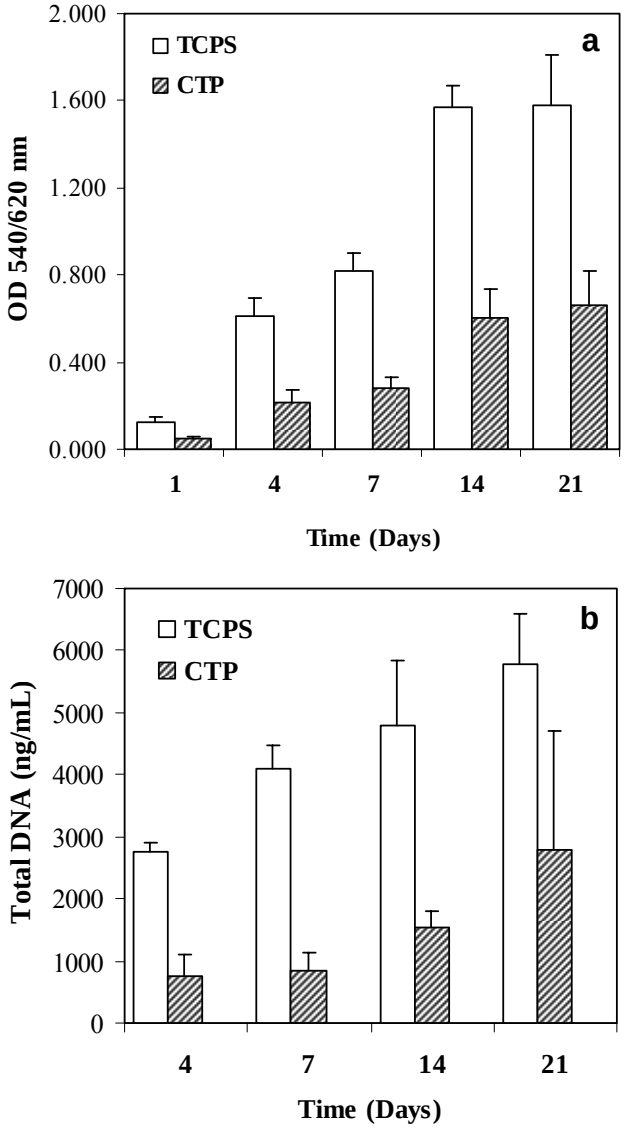

Figure 4. Cell proliferation on CTP microspheres as assessed by: (a) NR assay; (b) total DNA quantification. TCPS microcarriers were used as controls.

SEM images of bone marrow stromal cells cultured for 21 days on the surface of CTP and TCPS microspheres are presented in Fig. 5. The surface of CTP microspheres is almost completely covered by dense layers of cuboidal cells (Fig. 5a, 5b) that deposited an abundant amount of ECM, forming a three-dimensional fibril network (Fig. 5 b, 5c). The presence of ECM could already be detected at day 14 (data not shown). Cells grown on TCPS microcarriers (Fig. 5d) exhibit a flat, well-spread appearance and formed confluent monolayers.

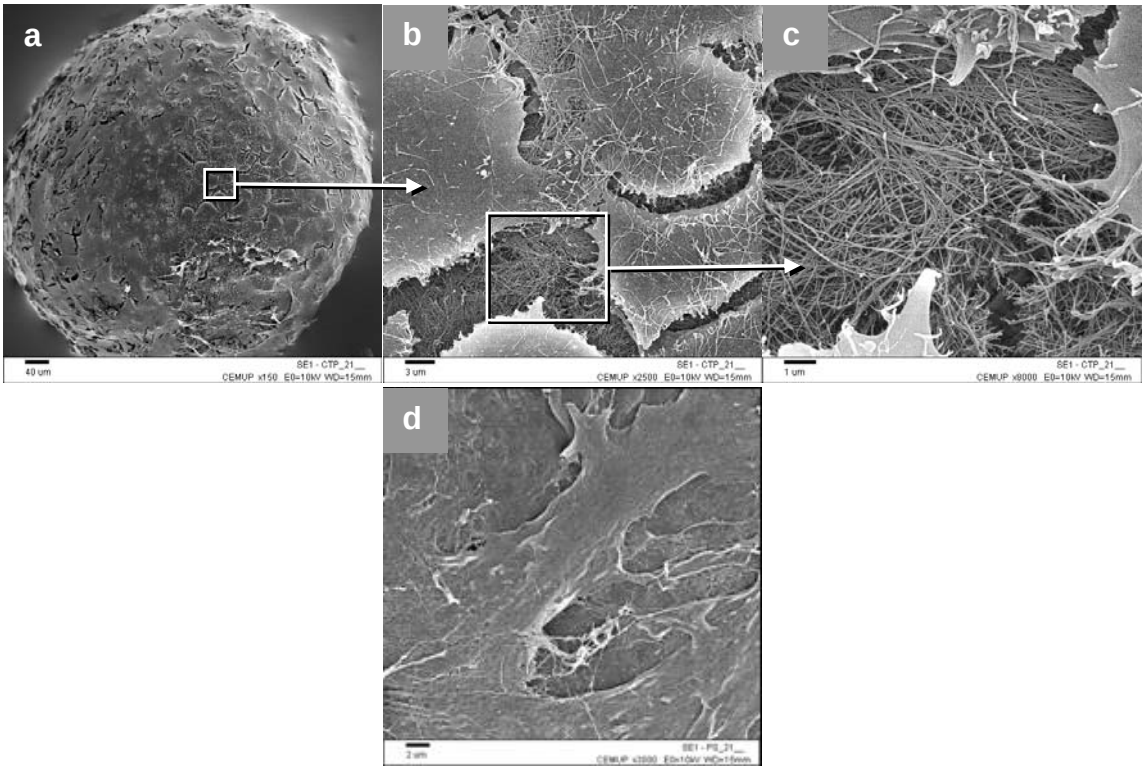

Figure 5. SEM images of bone marrow stromal cells cultured for 21 days on the surface of CTP microspheres, showing: (a, b) the substrate surface almost completely covered by dense layers of bone marrow cells; (b, c) a fibrillar ECM underlying cells. (d) SEM image of bone marrow stromal cells cultured for 21 days on the surface of a TCPS microsphere.

Cells were able to establish bridges between adjacent CTP microspheres, forming microsphere-cell clusters (Fig. 6).

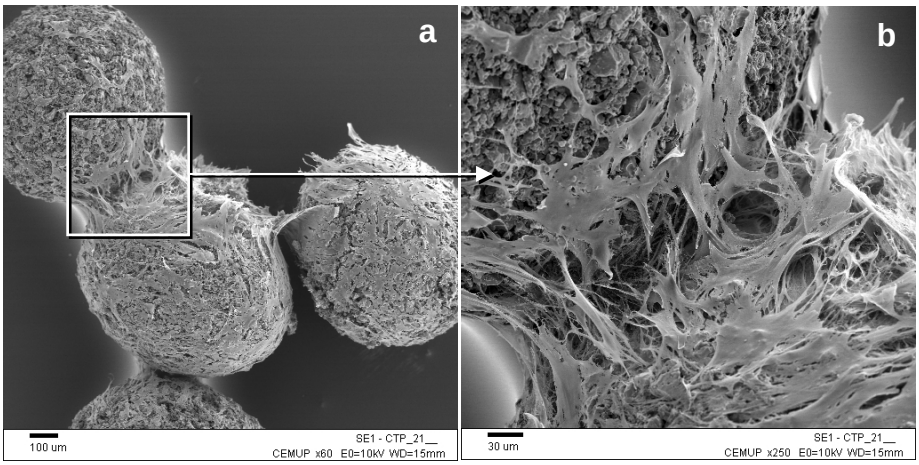

Figure 6. SEM images of bone marrow stromal cells cultured for 21 days on the surface of CTP microspheres, showing the establishment of cell bridges between adjacent microspheres.

Osteogenic differentiation

The expression of the osteoblastic phenotype was evaluated by monitoring ALP activity and OC secretion. Bone marrow stromal cells cultured on microspheres expressed ALP activity as assessed by histochemical staining and colorimetric analysis (Fig. 7). Temporal expression of ALP activity is depicted in Fig. 7a. As shown, ALP activity of cells cultured on CTP microspheres gradually increased along the first 2 weeks of culture, peaked around days 7-14, and then started to decrease. ALP levels were always significantly higher ($p<0.05$) than those of cells cultured on TCPS microcarriers. Histochemical ALP staining of microsphere-cell constructs after 7 days of culture (Fig. 7b) showed localised areas with intense staining for ALP (pink regions). OC secretion of cells cultured on CTP microspheres (Fig. 8) was higher at day 14 than at day 21 ($p=0.03$), and levels were significantly enhanced compared with cells grown on TCPS microcarriers ($p=0.03$).

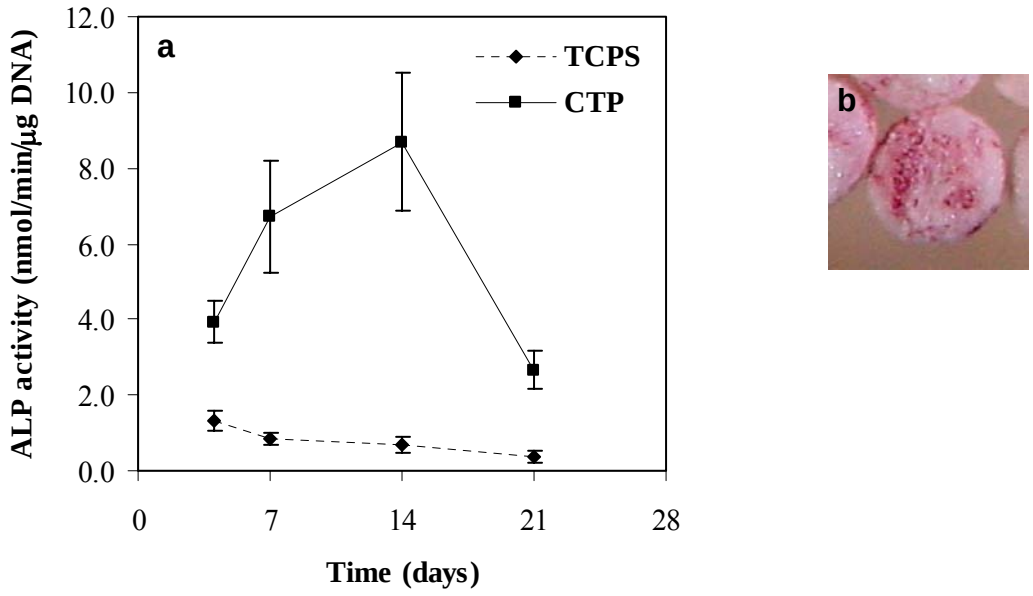

Figure 7. Expression of ALP activity by bone marrow stromal cells cultured on the surface of CTP microspheres as assessed by: (a) colorimetric analysis; (b) histochemical staining (pink areas). TCPS microcarriers were used as controls. Differences between days 7 and 14 are not statistically significant, but differences between the two materials are always statistically significant (p<0.05).

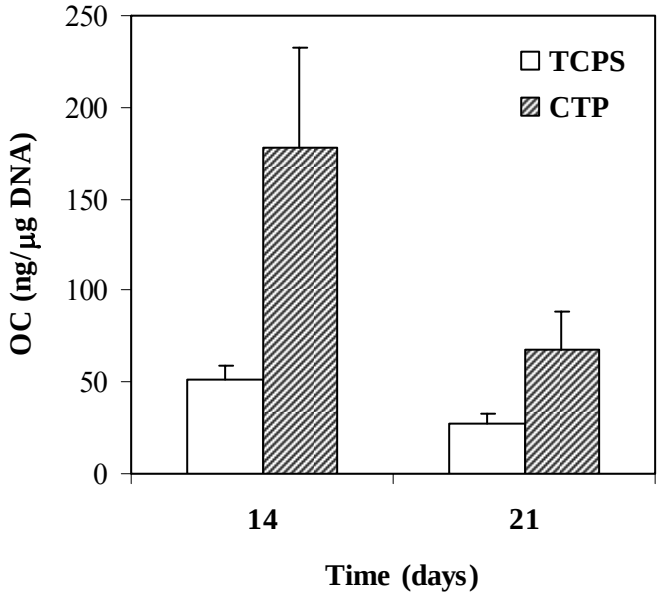

Figure 8. OC secretion by bone marrow stromal cells cultured on the surface of CTP microspheres at days 14 and 21. TCPS microcarriers were used as controls. Differences between the two materials, and between days 14 and 21 for each material, are statistically significant (p=0.03).

DISCUSSION

In this study, CTP microspheres were investigated for their ability to support adhesion, growth, and osteogenic differentiation of bone marrow stromal cells *in vitro*.

The methodology used to prepare CTP microspheres has been previously reported.[9,10] One of the most important advantages of this method is that it allows the preparation of spherical-shaped particles with an adequate and uniform size. It has been previously suggested[13] that the size and shape of microparticles for bone regeneration have a critical role in new bone formation by dictating the relative arrangement of particles within the implant site. Although some empty space is necessary to allow tissue and capillary ingrowth, excessive interstitial space, associated with the use of very large particles, may favour the development of fibrous tissue and compromise adequate healing. In addition, large particles are more difficult to inject, leading to a more invasive implantation. On the other hand, very small particles, as well as particles with broad size distributions, result in dense packing at the defect, which may impair tissue ingrowth and vascularization,[13] and some may be able to migrate from the implant site and cause injury elsewhere. Some authors found superior bone formation around particles measuring 0.3–0.6 mm, and demonstrated that larger and smaller particles were associated with less bone formation.[13,14] The shape of the microparticles is equally important. Irregularly shaped particles have sharp edges that are more susceptible to wear and may damage the surrounding tissue once implanted and/or induce strong inflammatory responses.[15] For all the above reason, it seams that spherical-shaped particles with a uniform and adequate size may present some advantages compared with other type of microparticles described in the literature.

Cell-material interaction studies were performed using rat bone marrow stromal cells. Bone marrow has long been recognised as a source of progenitor stromal cells that can be induced to differentiate along the osteoblastic lineage if cultured under appropriate conditions.[11,16-18] The development of osteoblastic cells from bone marrow stromal precursors is characterised by a sequence of events, involving cell adhesion, proliferation and expression of osteogenic differentiation markers, which are related to the synthesis, deposition and subsequent mineralization of a collagenous ECM.

The initial phase of the culture is probably the most critical stage, because the ability of cells to attach, adhere and spread will influence their capacity to proliferate and differentiate in

contact with the material.[19] In a microparticulate culture, the percentage of attached cells, in relation to the amount initially employed, is generally lower than the one obtained using flat surfaces, and the number of cells per microsphere is likely to be distributed over a range. To increase the possibility of cell-microsphere effective collisions, microspheres were inoculated with cells suspended in the smallest possible volume, because often proliferation will only occur if there is a sufficient density of attached cells per microsphere. The percentage of adherent cells after 24 h of culture was lower on CTP microspheres (16%) that on TCPS microcarriers (46%). Despite the lower percent of adhesion, CLSM images of the microsphere-cell constructs after 5 days of culture revealed that CTP microspheres were uniformly colonised by cells, which underwent cytoskeleton reorganisation, spread on the surface, and adopt a typical osteoblast-like morphology. Moreover, differences in cell adhesion apparently did not have an effect on growth and activity, as cells proliferated on both substrates at a similar rate, increasing approximately 12-fold in relation to day 1 in 2 weeks, and produced an abundant amount of fibrillar ECM. The results presented in this study further demonstrated that cells cultured on CTP microspheres are able to differentiate, and that ALP activity and osteocalcin secretion levels are significantly enhanced when compared with cells cultured on TCPS microcarriers.

The two substrates differ in terms of size, chemical composition and surface roughness, all of which are likely to influence protein adsorption and ultimately cell behaviour in contact with the materials.[20,21] In particular, CTP microspheres present a surface rougher than the one of TCPS microcarriers, which may partially explain our results, because it has long been known that cell attachment, proliferation and differentiation are sensitive to surface microtopography.[22,23] Several authors have reported altered attachment and enhanced differentiation of osteoblast-like cells on rough surfaces in comparison with smother ones, and suggested that structural features of the surface modulate the expression of phenotypic markers and influence the way cells respond to regulatory factors.[21,22,24] On smooth surfaces, osteoblasts-like cells are able to attach and proliferate but generally assume a more flattened fibroblastic appearance, and express relatively low levels of osteogenic markers.[22]

Regarding the chemical composition of the cell culture substrate, several reports on the positive effects of different types of calcium phosphates on osteogenic differentiation may be found in the literature, and it has already been reported that osteoblastic cells grown on these materials express increased ALP activity and/or OC levels compared with plastic surfaces.[25-27]

For the particular case of CTP, Knabe *et al*.[28] examined the effect of this calcium phosphate on the expression of bone-related genes and proteins by human bone-derived cells, and compared it with that of titanium and HAp-coated titanium. They showed that CTP had a more pronounced effect on osteoblastic differentiation, because it induced a greater expression of an array of osteogenic markers than that recorded for cells cultured on the other materials.

CONCLUSIONS

Bone marrow stromal cells were cultured for up to 21 days on CTP microspheres. Cells were able to attach, adopt a typical osteoblast-like morphology, and gradually proliferate along 2-3 weeks of culture. SEM images showed that the substrate surface becomes almost completely covered by dense layers of bone cells and a fibrillar ECM. Cells expressed ALP activity and secreted OC, which confirmed that differentiation along the osteoblastic lineage occurred.

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Chapter VIII

Adsorption of a therapeutic enzyme to self-assembled monolayers: effect of surface chemistry and solution pH on the amount and activity of adsorbed enzyme*

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ABSTRACT

The adsorption of a therapeutic enzyme to self-assembled monolayers (SAMs) of different functionalities (X= CH_3-, OH- and COOH-) was evaluated as a function of solution pH. Radiolabelling studies showed that the enzyme has higher affinity for hydrophobic than for hydrophilic surfaces, and that highest adsorption was obtained at the more acidic pH values (4.5 and 5.5), irrespective of the type of surface. IRAS and XPS measurements confirmed this tendency. Dye-binding studies and fluorescence quenching were used to investigate if a pH variation induces any conformational changes on the enzyme. Both methods suggest that lowering the pH from physiological to acidic values triggers an increased exposure of non-polar sites in the enzyme, which may modulate its adsorption behaviour to the more hydrophobic surfaces. At pH 4.5 the enzyme carries a substantial positive net charge and therefore relatively low native-state stability. As a consequence, surface-binding may be

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favoured, irrespective of the type of surface, by providing increased conformational entropy to the enzyme. The specific activity of the adsorbed enzyme was strongly dependent on the conditions used. A decrease in specific activity (ca. 30 % of control) was observed after adsorption on CH_3-SAMs for all the pH tested. Adsorption on gold and on the more hydrophilic SAMs (OH- and COOH-), resulted in different degrees of inactivation at the more acidic pH (4.5), and in enzyme activation (up to ca. 230% of control) at higher pH (7-8), near the isoelectric point of the enzyme.

Keywords: Adsorption, enzyme, self-assembled monolayers (SAMs), enzyme activation

INTRODUCTION

Adsorption of proteins and their interaction with surfaces has been the subject of considerable research in the biomedical field. Numerous proteins, including several enzymes, have been immobilised through adsorption on different supports to be used in a wide range of therapeutic and diagnostic applications. In drug delivery, controlled protein adsorption has been used as a means to temporarily couple the protein to the vehicle.[1] Reversibility of binding and the retention of an active conformation of the protein upon immobilisation and subsequent release are essential requirements. On the other hand, the proper transport and delivery of entrapped proteins from polymeric microcarriers, for example, is also intimately linked with protein adsorption. In this case, however, the extent of unspecific and irreversible adsorption of the protein to the matrix should be reduced as much as possible, since a fraction of the total entrapped amount will consequently become unavailable, leading to an incomplete release profile and wastage of valuable protein.[2] The selection of the most adequate delivery matrix for a certain application could clearly benefit from previous knowledge on the adsorption behaviour of the protein of interest.

The process of protein adsorption is complex and is affected by various factors. The rate and extent of adsorption will be determined by the properties of the protein and its concentration in solution, by the characteristics of the adsorption matrix, the nature of the solvent and other properties of the medium, namely pH, ionic strength and temperature.[3] The most relevant phenomena involved in the process of protein adsorption are electrostatic and hydrophobic interactions and protein's intrinsic structural stability.[3] Upon

adsorption, proteins generally undergo structural rearrangements dependent on the type and strength of the interactions established with the matrix.[4,5] Although conformational alterations may result on protein denaturation and/or loss of functional activity,[6,7] in some situations they can be beneficial rather than detrimental. For example, enzyme stability is often better retained in immobilised formulations than on native free forms[5, 8] and enzymatic activity can be enhanced in the presence of an interface.[9,10]

The mechanistic aspects of protein interaction with surfaces can be investigated using self-assembled monolayers (SAMs) of long-chain alkanethiols on gold [$HS(CH_2)_nX$].[11] These highly ordered model surfaces can be prepared by exposing different functional groups (X) on the surface allowing a wide range of chemical compositions and wettabilities to be explored.[12] The objective of the present study was to investigate some fundamental aspects of the interaction of glucocerebrosidase (GCR) with surfaces using SAMs of different functionalities (X= CH_3, OH and COOH).

The activity and/or stability of lysosomal GCR is defective in patients with Gaucher disease, the most prevalent metabolic storage disorder, which is characterised by a number of severe disabling symptoms including bone lesions.[13] For enzyme replacement therapy (ERT), recombinant GCR is administered intravenously and is internalised by target cells by mannose receptor mediated endocytosis.[13] Although ERT seems to be efficient in reverting most of the symptoms, the delayed and modest skeleton response remains the major issue in the overall effectiveness and management of affected patients.[13,14] This is indicative that adjuvant therapies are necessary to restore bone function more rapidly and/or to a greater extent than with ERT alone.[13] The development of a vehicle for site-specific GCR delivery to bone resident Gaucher cells is currently under investigation in our group,[15] as a therapy that may be used in association with ERT.

In this study, radiolabelling was used to quantify the amount of GCR adsorbed onto SAMs as a function of solution pH. Fourier transform infrared reflection absorption spectroscopy (IRAS) and X-ray photoelectron spectroscopy (XPS) were used as alternative semi-quantitative techniques. This information was complemented with data on the catalytic activity of adsorbed GCR, with the aim of providing insight on the biological status of the adsorbed enzyme. Fluorescence studies were performed to investigate if a pH variation induces conformational changes on the enzyme.

MATERIALS AND METHODS

Preparation and characterisation of SAMs

Preparation of gold substrates and monolayer formation

Gold substrates were prepared as previously described.[16] Briefly, chromium (5 nm) and gold (25 nm) films were deposited by ion beam sputtering onto silicon wafers (polished/etched; crystal orientation <100>; AUREL Gmbh). The Cr layer was used to improve adhesion of gold to silicon. Wafers were coated with 1.5 μm of photoresist (PFR7790EG, JSR Electronics) to protect the film surface, and diced into pieces (0.5×0.5 and 1×1 cm^2) using a DISCO DAD 321 automated saw.

The following alkanethiols (Aldrich): 11-mercapto-1-undecanol [SH-$(CH_2)_{11}$OH; 97%], 1-undecanethiol [SH-$(CH_2)_{10}CH_3$; 92%] and 11-mercaptoundecanoic acid [SH-$(CH_2)_{10}$COOH; 95%] were used as received. Alkanethiol solutions of 1 mM in ethanol (99.8%, Merck) were prepared under N_2 in a glove box. Immediately prior to use, gold substrates were cleaned twice in acetone, rinsed with ethanol and immersed in a "piranha" solution (7 parts of H_2SO_4 and 3 parts of 30% v/v H_2O_2) for 10 min (Caution: this solution reacts violently with many organic materials and should be handled with care). Substrates were sequentially rinsed with ethanol/distilled-deionised water/ethanol for 2 min in an ultrasonic bath, and then dried with Ar. Subsequently, they were immersed in alkanethiol solutions and incubated at room temperature for 24 h in N_2. SAMs were finally washed 3 times in ethanol in an ultrasonic bath for 2 min, dried and maintained under Ar until used.

Contact angle measurements

Contact angle (θ) measurements were performed by the sessile drop method (Data Physics OCA 15, equipped with video CCD-camera and SCA 20 software), using an electronic unit with a gas tight dosing syringe (Hamilton). SAMs were placed in a closed, thermostated chamber (25º C) saturated with water to prevent evaporation. Measurements were carried out using distilled-deionised water (4 μL drops, n=3). After drop deposition, images were acquired every 2 s over 600 s. Droplet profiles were fitted using different mathematical

functions in order to calculate θ. The Young-Laplace method was used for θ≥90º; the ellipse method for 30º<θ<90º, and the tangent method for θ<30º. Water contact angles (θ_{water}) were calculated by extrapolating the time dependent curve to zero.

XPS measurements

Measurements were carried out on a VG Scientific ESCALAB 200A (UK) spectrometer using magnesium Kα (1253.6 eV) as radiation source. The photoelectrons were analysed at a take-off angle normal to the interface. Survey spectra were collected over a range of 0–1150 eV with analyser pass energy of 50 eV. High-resolution C1s, O1s, S2p, N1s and Au4f spectra were collected with analyser pass energy of 20 eV. Spectra were fitted with XPSPEAK (Version 4.1) software.

Preparation and characterisation of GCR solutions

Preparation of enzyme solutions

Enzyme stock solutions (2 mg/mL) were prepared by reconstituting recombinant human glucocerebrosidase lyophilised powder (GCR; Genzyme Corporation) in distilled-deionised water. Stability of CGR in the stock solution is maintained for more than one month at 4ºC. The final concentration of GCR solution was confirmed by the bicinchoninic acid assay using bovine serum albumin (BSA) as a standard. The stock solution was diluted to 0.1 mg/mL immediately prior to use, in 50 mM citrate buffer containing 0.154 M NaCl and 0.01 M NaI. The pH was adjusted after GCR addition to 4.5, 5.5, 7.0 and 8.0. pH values were always checked for stability after measurements.

Activity of GCR after incubation at different pH

GCR was pre-incubated at pH 4.5, 5.5, 7.0 and 8.0 for up to 60 min at room temperature. Aliquots were obtained periodically and GCR activity was assayed as the hydrolysis of the water-soluble substrate 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-Glc; Glycosynth), as described elsewhere.[17] The reaction mixture consisted of 0.06% v/v Triton

X-100, 5 mM 4-MU-Glc and 50-100 mM citrate-phosphate buffer (pH 5.5) with or without 6 mg/mL of Na-taurocholate. After incubation for 30 min at 37ºC the reaction was stopped with 1 M glycine in 30% NaOH (pH 10.3). The product 4-methylumbelliferone (4-MU) was quantified by fluorescence spectroscopy with excitation at 366 nm and emission at 445 nm. GCR activity is reported as nmol 4-MU/mL/h.

Fluorescence spectroscopy

Analyses were carried out in a Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc.). Quenching studies were performed by recording fluorescence emission spectra of GCR at different pH, in the absence or presence of 0.1 M NaI. Excitation was set at 295 nm and emission monitored in the range 310-400 nm with slit widths of 10 nm. Dye-binding studies were performed by recording fluorescence emission spectra of 8-anilino-1-naphtalene sulfonic acid (ANS, 20 μM) at different pH, in the absence or presence of GCR. Excitation was set at 370 nm and emission monitored in the range 400-600 nm with slit widths of 10 nm. Spectra were recorded 2 min after GCR addition.

Adsorption of GCR to SAMs and enzymatic activity assay

SAMs with varying functionalities (X= OH, COOH and CH_3) were placed in 24-well tissue culture plates (Sarsted) with the surface facing up. Depending on the method of quantification subsequently used, incubation with GCR solutions (0.1 mg/mL) at different pH was carried out by placing a drop of solution on the top of each sample (radiolabelling studies), or by completely immersing the samples in 500 μL of GCR solution (IRAS and XPS studies). When drops were used, saline (0.154 M NaCl) was added to the periphery of the wells to avoid evaporation. Adsorption tests were carried out at room temperature for 30 min. A concentration of 0.1 mg/mL and an adsorption time of 30 min were selected based on other studies, previously reported by the authors and in the literature,[16] concerning the adsorption behaviour of other proteins, namely albumin, which has approximately the same size of the enzyme used in the present investigation (ca. 60 kDa). These previous studies with albumin, using SAMs with the same functional groups that those used in the present investigation, have demonstrated that a concentration of 0.1

mg/mL induces the formation of a monolayer of albumin, and that higher concentrations may promote multilayer adsorption. Furthermore, kinetic studies demonstrated that an adsorption time of 30 min is sufficient to attain steady-state adsorption values, even when protein concentrations lower than 0.1 mg/mL are used.[16]

After GCR adsorption, the solution was withdrawn and SAMs were rinsed three times with saline. The amount of adsorbed GCR was quantified using different techniques as described bellow. SAMs incubated under the same conditions in buffer solutions without GCR were used as controls.

For enzymatic activity measurements, SAMs with adsorbed ^{125}I-GCR were immediately incubated with the substrate and the catalytic activity assayed as described above but in the absence of detergents. At the end of the reaction, SAMs-associated radioactivity was counted and the product (4-MU) quantified. The GCR concentration per assay was kept constant. Free-GCR was used as a control. GCR activity is reported as nmol 4-MU/h/mg GCR (specific activity).

Quantification of adsorbed GCR

Radiolabelling measurements

^{125}I-labelled GCR was prepared according to the Iodogen method.[18] ^{125}I-GCR was purified in a pre-packed PD-10 Sephadex G-25 M column (Amersham Pharmacia Biotech) previously blocked with 20% w/v BSA and equilibrated in phosphate-buffered saline (pH 7.4). The final solution contained only trace amounts of free ^{125}I (<2%), as estimated by trichloroacetic acid precipitation (TCA, 10% v/v). ^{125}I-GCR was combined with non-labelled GCR solutions (10^8 cpm/mg) and used immediately.

The amounts of adsorbed GCR were estimated by counting SAMs-associated radioactivity in a γ-counter, after exposure to GCR solutions. Surface concentration was calculated by the equation:

$$GCR(mg/m^2) = \frac{Counts(cpm)}{A_{solution}(cpm/mg) \times SA(m^2)}$$

where, *Counts:* SAMs-associated radioactivity, $A_{solution}$*:* specific radioactivity of GCR solution and *SA:* surface contact area of the drop (calculated with the contact angle measuring system software).

Tests were carried out in the presence of 10 mM NaI to suppress the adsorption of residual free ^{125}I.[19] To test the effectiveness of this measure, adsorption of ^{125}I (10^7 cpm/mL) to OH- and CH_3-SAMs at pH 4.5 and 8.0 was analysed. The effect of radiolabelling on GCR adsorption behaviour was assessed by quantifying GCR adsorption on gold (n=4) using a series of solutions of constant GCR concentration (0.1 mg/mL) but of varying (10-50%) labelled-to-unlabelled GCR ratios.[19]

XPS and IRAS measurements

For XPS and IRAS measurements, samples with adsorbed GCR (CH_3- and OH-SAMs, pH 4.5 and 8.0) were dried with Ar before analyses. IRAS measurements were performed on a FTIR spectrophotometer (Perkin Elmer 2000), coupled with VeeMax II Accessory (PIKE) and a liquid N_2-cooled MCT detector. Spectra were collected using the 80º grazing angle reflection mode. The incident light was p-polarised. Samples were analysed with 200 scans and 4 cm^{-1} resolution using a gold surface as the background. XPS measurements were performed as already described.

RESULTS

Characterisation of SAMs

The chemical composition of SAMs was analysed by XPS. Survey spectra (data not shown) reveal only the presence of the expected monolayer elements (Au, C, S in all SAMs; and also O in OH- and COOH-SAMs), indicating the absence of contamination. Surface atomic compositions of the three types of SAMs calculated from the XPS high-resolution spectra are in accordance with those described elsewhere, and for that reason were not included here.[20]

The θ_{water} found for the gold surfaces was 64 ± 2º. Similar values were obtained by other

authors.[20,21] Although a clean gold surface has been described as hydrophilic, after some minutes of exposure to the laboratory atmosphere, its θ_{water} significantly increases due to adsorption of non-polar contaminants.[22] However, after 24 h of immersion in the thiol ethanolic solutions, the effect of the contaminant layers on the gold surface is negligible.[21] The θ_{water} measurements indicate that CH_3-SAMs are hydrophobic (107º ± 0.4), while the SAMs with OH- and COOH- functionalities are hydrophilic (19º ± 2.1 and 38º ± 0.9, respectively), in accordance with data obtained previously.[20]

Characterisation of GCR solutions

Activity of GCR after incubation at different pH

Catalytic activity of GCR following incubation at different pH was assayed in the presence or absence of taurocholate, a well-known activator of the enzyme.[17] When the detergent is absent GCR activity is low, while in its presence GCR is stimulated to a great extent (ca. 30-fold). Both assays demonstrated that under the conditions used, the catalytic activity of GCR is maintained for at least 60 min or, if any alteration occurred during incubation, it may be assumed that it is reverted under the assay conditions. However, both assays show that the catalytic activity of GCR following incubation at pH 4.5 is lower compared to other pH.

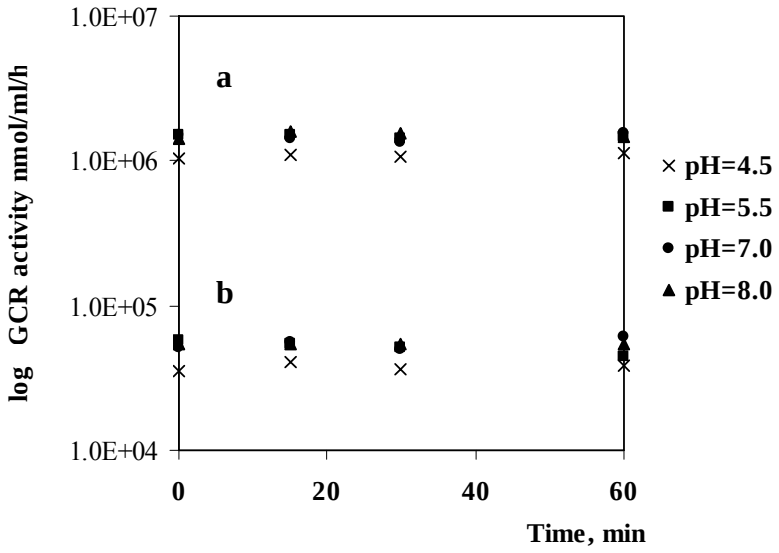

Figure 1. Catalytic activity (n=5) of GCR after pre-incubation at different pH for up to 60 min. Assays were performed at pH 5.5 in the absence of activators.

Fluorescence spectroscopy

The intrinsic fluorescence of GCR derives mainly from its 12 tryptophan residues.[23] No significant changes in the maximum emission wavelength (≈ 336 nm) were observed on GCR fluorescence emission spectra when the pH was varied from 8.0 to 4.0 (data not shown). Similar spectra were acquired in the presence of an aqueous quencher (0.1 M NaI) to probe the degree of exposure of non-polar tryptophan residues to the aqueous environment. Fig 2. shows the efficiency of quenching ($1-F/F_0$) as a function of pH, where F_0 and F are the fluorescence intensities at 336 nm before and after addition of the quencher, respectively. The higher quenching effect was detected at pH 4.0 ($1-F/F_0=0.76$), and the efficiency of quenching decreased as the pH increased. No significant differences were observed between pH 7.0 and 8.0 ($1-F/F_0 \approx 0.38$). As a complementary method to detect subtle changes in GCR conformation as a function of pH, dye-binding experiments with the fluorescent probe ANS were performed. The fluorescence of ANS can be considerably enhanced upon interaction with exposed hydrophobic sites on proteins, and is frequently used as an indicator of relative surface hydrophobicity.[24] Fig 3. shows the GCR-dependent ANS fluorescence emission spectra at different pH, and reveals a trend towards increasing fluorescence intensity as a function of decreasing pH. A modest blue-shift in the emission maximum also occurs and increases when the pH is lowered. Both methods suggest that lowering the pH to more acidic values triggers an increased solvent exposure of non-polar sites in the enzyme.

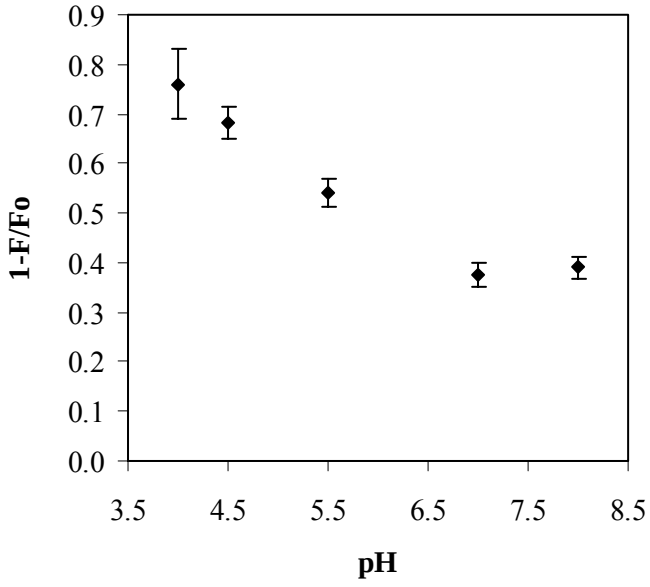

Figure 2. Efficiency of quenching ($1-F/F_0$) by 0.1M NaI as a function of pH. F_0 and F are the fluorescence intensities at 336 nm before and after addition of the quencher, respectively. Results are presented as mean ± standard deviations (n=3).

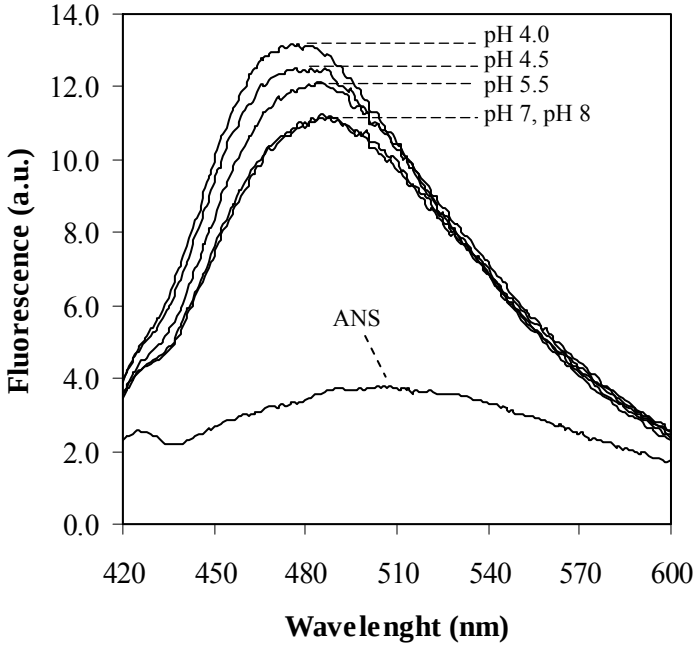

Figure 3. Effect of solution pH on ANS fluorescence emission intensity. Upper spectra were obtained after addition of GCR to ANS solutions at different pH. Results are presented as an average (n=3).

Quantification of adsorbed GCR

Radiolabelling measurements

The amounts of GCR adsorbed to gold and SAMs at different pH are presented in Fig. 4. In all the samples tested, GCR adsorption was pH dependent, with the highest adsorbed amounts being obtained at the more acidic pH (4.5). Moreover, adsorption was always higher on gold and hydrophobic SAMs (CH_3-) than on hydrophilic SAMs (OH- and COOH-).

For CH_3-SAMs, the amount of adsorbed GCR increased from 4.2 ± 0.2 mg/m^2 to 7.4 ± 0.2 mg/m^2 as the pH decreased from 8.0 to pH 4.5, respectively. GCR adsorption on gold followed a similar pattern. On OH-SAMs, the amount of adsorbed GCR attained the highest value (1.4 ± 0.4 mg/m^2) at the more acidic pH (4.5). However, no significant differences were observed between surface concentrations of GCR adsorbed at the other pH, which were all in the range of 0.5–0.6 mg/m^2. Finally, on COOH-SAMs, the adsorbed amounts were higher at acidic pH values, reaching 1.6 ± 0.3 mg/m^2 at pH 4.5 and 1.1 ± 0.2 mg/m^2 at pH 5.5; while a lower surface concentration of ca. 0.5–0.6 mg/m^2 was obtained for pH 7.0 and 8.0.

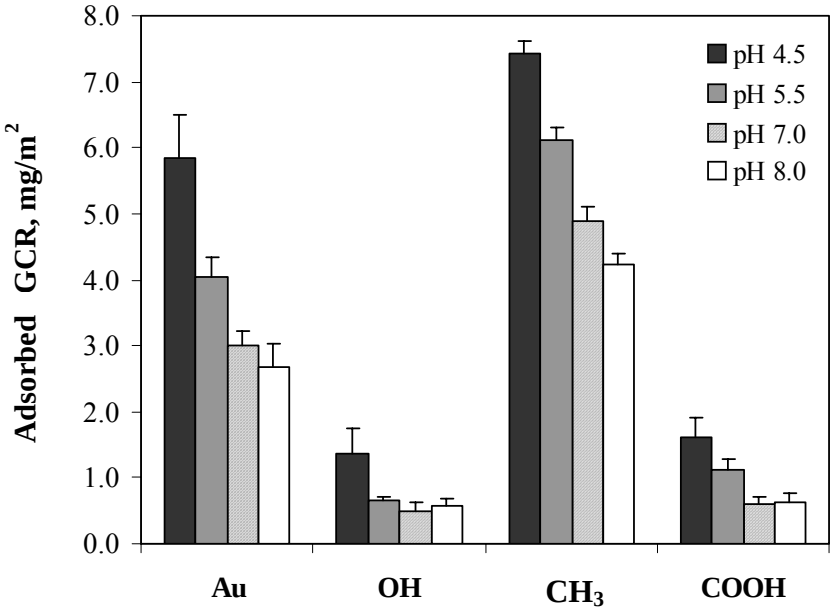

Figure 4. Amount of GCR adsorbed at different pH to SAMs of varying functionalities (X= OH, COOH and CH_3). Adsorption was carried out for 30 min at room temperature and ^{125}I-GCR was used as a tracer. Results are presented as mean ± standard deviations (n=5).

Effect of radiolabelling and of free ^{125}I on GCR adsorption

Assessment of the effect of radiolabelling on the adsorption behaviour of ^{125}I-GCR was carried out, since labelling may result in an alteration of the physicochemical and/or biological properties of the enzyme.[19,25] The test relies on the assumption that if the labelled and unlabelled enzyme forms have the same adsorption behaviour, surface coverage will only be dependent on the total GCR concentration. Results showed that irrespective of the labelled-to-unlabelled ratios used, the adsorbed amount was always ca. 5 mg/m^2, suggesting that there is no preferential adsorption of one of the forms.

Adsorption studies were carried out in the presence of NaI, which strongly reduces the adsorption of free ^{125}I.[19,26] In order to confirm the validity of this method when applied to our system, adsorption of ^{125}I to OH- and CH_3-SAMs at pH 4.5 and 8.0 in the presence of NaI was studied. Results showed that only ca. 0.2% of the total radioactivity present on each drop (7×10^4 cpm) becomes associated with the surfaces (≈ 120 cpm), irrespectively of the type of SAM or pH used. This demonstrates that during GCR adsorption the contribution of trace amounts of free ^{125}I to total SAMs-associated radioactivity is very low and can be neglected.

IRAS measurements

Proteins have characteristic vibrational absorption bands in the IR region. Amide I band (1690 ± 45 cm^{-1}) represents primarily the C=O stretching vibrations, while amide II band (1540 ± 60 cm^{-1}) represents the N-H in-plane deformation coupled with C-N stretching modes.[27]

Fig. 5 shows IRAS spectra of the amide bands (I and II) of GCR adsorbed onto OH- and CH_3-SAMs. The intensities of the bands were calculated with a baseline drawn from 1765 cm^{-1} to 1440 cm^{-1}. A higher amount of adsorbed GCR was detected in hydrophobic CH_3-SAMs than on hydrophilic OH-SAMs for both pH tested. On CH_3-SAMs, a higher amount of adsorbed GCR was detected at pH 4.5. Differences were more evident in the amide II region. In the case of OH-SAMs, GCR adsorption did not appear to vary with pH. The position of amide II bands of GCR adsorbed on OH-SAMs is shifted to lower frequencies (1515 cm^{-1}) compared to those of GCR adsorbed on CH_3-SAMs (1550 cm^{-1}). Since the shape and position of amide bands have been correlated with the presence of α-helical, β-sheet and random-coil domains, changes of these parameters may reflect alterations in protein secondary structures.

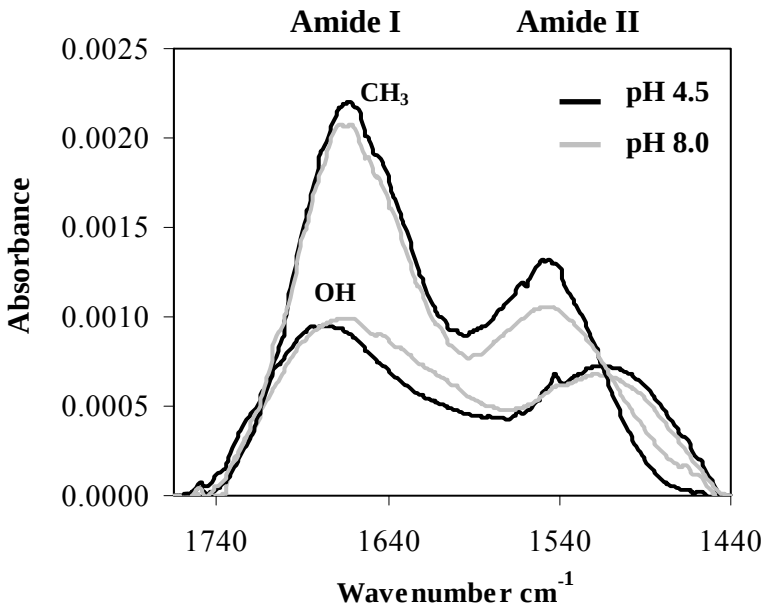

Figure 5. IRAS spectra in the amide I and amide II regions of GCR adsorbed on OH- and CH_3-SAMs. Adsorption was performed at pH 4.5 (black lines) and pH 8.0 (gray lines). Results are the average of two spectra.

XPS measurements

The relative amount of GCR adsorbed on OH- and CH_3-SAMs at pH 4.5 and 8.0 was followed by XPS. Differences in the atomic % of N1s and in the N1s/Au4f atomic ratio were calculated, since the N1s signal is specific for the protein and the Au4f signal is specific for the SAMs. Results are presented in Tab. I. The composition of SAMs without adsorbed enzyme (control) and the % of C1s, O1s and S2p in all samples were also included. Upon GCR adsorption, new elements are detected in the monolayers: N_2 in both SAMs, and also O_2 in CH_3-SAMs. The atomic % of C increases, while the atomic % of Au and S decreases (CH_3-SAMs) or remains practically unchanged (OH-SAMs). The atomic % of N1s as well as the N1s/Au4f atomic ratio are higher on hydrophobic CH_3-SAMs than on hydrophilic OH-SAMs. Within each type of SAM, both parameters varied with pH, with higher amounts being detected at pH 4.5. This is well illustrated in the N1s high-resolution XPS spectra (Fig. 6) by the relative intensity of the nitrogen peak (≈ 400 eV): CH_3 (4.5) > CH_3 (8.0) > OH (4.5) > OH (8.0). Fig. 7 shows that changes in the N1s/Au4f ratio as a result of varying the functional group of SAMs and solution pH are directly correlated (r^2=0.987) with the mass per unit area of adsorbed GCR, as estimated by radiolabelling.

Table 1. Atomic percentages (C1s, O1s, S2p, N1s and Au4f) obtained by XPS of OH- and CH_3-SAMs, before and after GCR adsorption at pH 4.5 and pH 8.0.

Atomic	**CH_3**			**OH**		
%	**Control**	**pH 4.5**	**pH 8.0**	**Control**	**pH 4.5**	**pH 8.0**
C_{total}	35.4	46.5	40.6	30.9	32.2	36.0
N_{total}	–	6.5	5.0	–	1.6	0.9
O	–	6.5	6.8	8.9	5.1	2.6
S	2.7	2.3	2.6	3.1	2.8	3.1
Au	61.9	38.2	44.9	57.2	58.3	57.3
N_{total}/Au	–	0.170	0.111	–	0.027	0.016

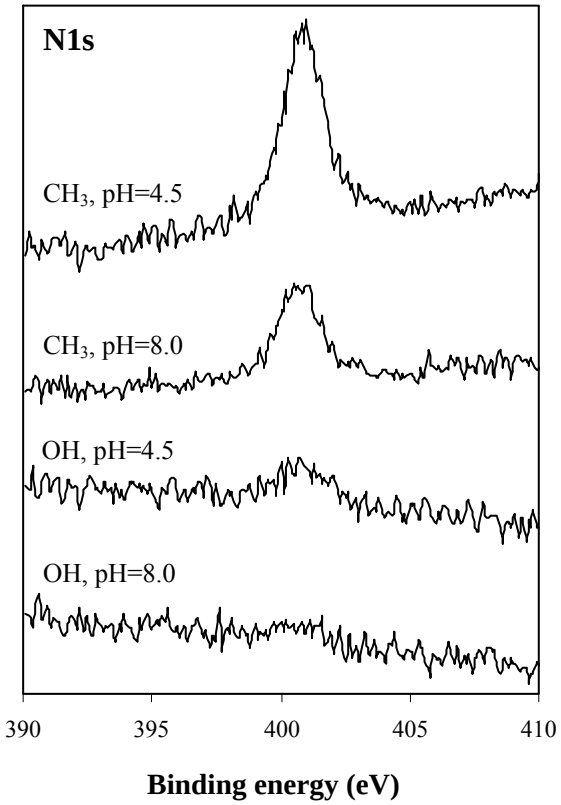

Figure 6. N1s high-resolution XPS spectra for CH_3- and OH-SAMs, after GCR adsorption at pH 4.5 and pH 8.0.

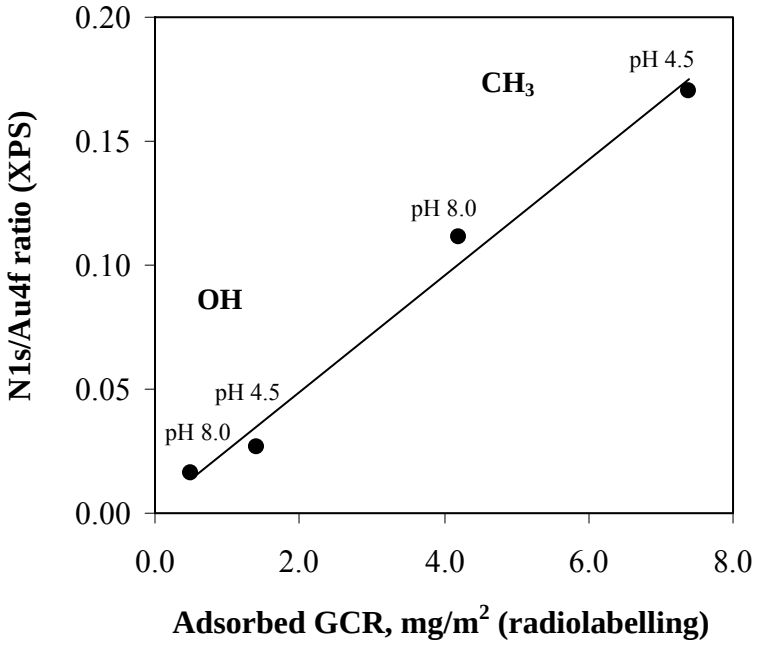

Figure 7. Correlation of the surface concentration of GCR (mg/m^2) estimated by radiolabelling with the N1s/Au4f atomic ratio obtained by XPS.

Fig. 8 represents the C1s high-resolution XPS spectra of CH_3-SAMs before (Fig. 8a) and after (Fig. 8b) GCR adsorption (pH 4.5). These are presented as an example, to illustrate the appearance of new carbon peaks due to GCR adsorption. On control SAMs (Fig. 8a), the C1s spectra showed the characteristic peak of C-C/C-H bonds with a binding energy around 285.0 eV. On samples containing adsorbed GCR (Fig. 8b) broadening of C1s peak was observed. Peak-deconvolution was made considering the contribution of 4 peaks,[28] namely: C−C/C−H bonds (hydrocarbon) at 285.1 eV, C-N (amine) at 286.2 eV, O=C−N (amide) at 287.9 eV and O−C=O (carboxyl) at 288.9 eV.

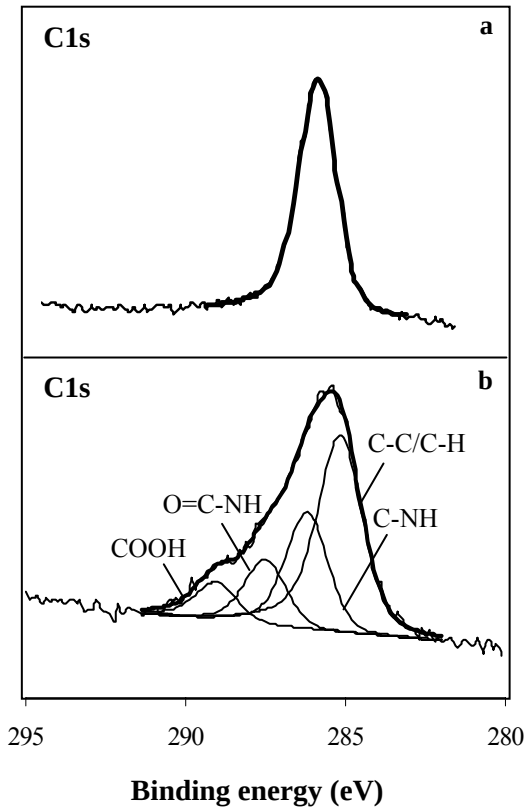

Figure 8. C1s high-resolution XPS spectra (resolved into component peaks) for CH_3-SAMs, before (a) and after (b) GCR adsorption at pH 4.5.

Catalytic activity of adsorbed GCR

Fig. 9 shows the specific activity of GCR adsorbed on SAMs at different pH. specific activity. of free GCR following incubation at different pH is also depicted as a control (pH 4.5: dashed line, other pH: solid line). The catalytic activity of adsorbed GCR was strongly

depended on the type of surface used. Higher activities were obtained on gold and on the more hydrophilic samples (OH- and COOH-SAMs) than on hydrophobic CH_3-SAMs. For GCR adsorbed on CH_3-SAMs, the specific activity was always lower than that of the control (ca. 30 %) and a pH-dependence was not evident. The specific activity of GCR adsorbed to the other surfaces was pH dependent, increasing as the adsorption pH increased. When GCR was adsorbed at pH 7.0-8.0 its specific activity was higher than that of the control. GCR adsorbed on OH-SAMs exhibited the highest specific activity (ca. 230 % of control).

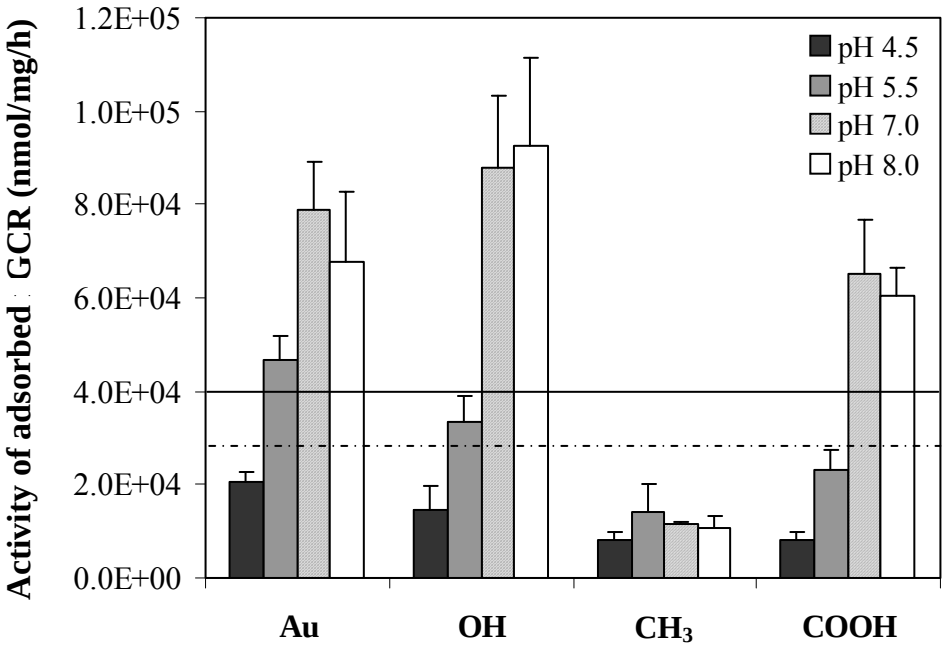

Figure 9. Specific activity of GCR adsorbed at different pH to SAMs of varying functionalities (X= OH, COOH and CH_3). The specific activity of free-GCR following incubation at pH 4.5 (dashed line) and at the other pH (solid line) was used as a control. Results are presented as mean ± standard deviations (n=5).

DISCUSSION

The objective of the present work was to investigate some fundamental aspects of the behaviour of the lysosomal enzyme glucocerebrosidase (GCR) at a well-defined solid-

liquid interface. GCR is a water-soluble glycoprotein with 497 amino acids, a molecular weight around 60 kDa and an isoelectric point (IP) around 7.[29] It has four Asn-linked oligosaccharide structures, from which the terminal sialic acid, galactose and N-acetyl-glucosamine sugars are removed during manufacturing.[29] The modified oligosaccharides consist primarily (ca. 95%) of neutral species and contribute to approximately 6% of the total molecular weight.[29] It has been ascertained that GCR has substantial hydrophobic properties.[23,30]

Adsorption studies were performed using SAMs of different functionalities (X= CH_3-, OH- and COOH-) and different solution pH (pH= 4.5, 5.5, 7.0, 8.0). The extent of GCR adsorption was analysed by radiolabelling. IRAS and XPS were used as alternative semi-quantitative techniques.

Consistent with the data reported by several authors for other proteins,[3,31] maximal GCR adsorption was observed on gold and hydrophobic CH_3-SAMs (θ_{water}= 107º). The extent of adsorption on this type of surface was strongly influenced by pH. However, in contrast to what is generally observed for several proteins, maximal adsorption was not observed at the enzyme IP (≈7). Instead, there was a gradually higher affinity for adsorption on gold and CH_3-SAMs as the pH decreased. Since adsorption on these surfaces is likely to be initiated by hydrophobic interactions, it is reasonable to assume that GCR surface hydrophobicity dictates, at least partially, its adsorption behaviour.[3] Therefore, based on studies with other systems,[24,32,33] we hypothesised that lowering the pH from physiological to acidic values may trigger an increased solvent exposure of non-polar sites in the enzyme, thus promoting surface-binding. To investigate this, the effect of solution pH on GCR tryptophan fluorescence was evaluated. Maximum wavelength (λ_{max}) shifts and changes in fluorescent intensity are related with alterations in the local environment of tryptophan residues, and are often used in protein structural analyses.[34] The fluorescence emission spectra of GCR showed a λ_{max} around 336 nm, but no significant changes were detected in its position over the pH range 4-8, which suggests that any pH-induced alterations in GCR must be subtle, without affecting its overall intrinsic fluorescence. In order to be able to detect eventual small changes on GCR conformation, complementary fluorescence studies were performed. GCR tryptophan fluorescence showed an increased sensitivity to external polar quenching by I^- at lower pH, which is indicative of an increased exposure of non-polar tryptophan residues to the aqueous environment.

Similarly, ANS-binding experiments revealed an increased solvent accessibility of hydrophobic sites as a function of decreasing pH. Taken together these results correlate with the enhancement of surface-binding to more hydrophobic surfaces at lower pH.

The specific activity of GCR upon adsorption to CH_3-SAMs decreased significantly (≈ 30% of control). This was not unexpected, since in general proteins change their conformation to a great extent upon adsorption to hydrophobic surfaces.[3,4] Loss of activity upon adsorption is not, however, proof of conformational change in itself, since a considerable degree of loss might be expected, even with complete retention of the enzyme structure, due to an inadequate orientation of the active site at the surface. Clustering of enzyme molecules at the surface, may also lead to a decreased substrate-accessibility of the active site and hence a decreased specific activity.

Adsorption of GCR to OH- and COOH- SAMs (θ_{water}= 19º and 38º respectively) was significantly lower. Nevertheless, higher adsorption was also observed at the more acidic pH (4.5). The intrinsic structural stability of the enzyme may be the major driving force for adsorption under such conditions. At pH 4.5, which is considerably below its IP, GCR carries a substantial positive net charge and therefore relatively low native-state stability.[3] As a consequence, surface-binding may be favoured, irrespective of the type of surface, by providing increased conformational entropy to the enzyme. The lower activity of free GCR following incubation at pH 4.5, compared to that at other pH, seems to corroborate this observation. In addition, the specific activity of GCR when adsorbed at acidic pH is always lower, especially at pH 4.5, probably result7ing from a combination of multipoint surface-interactions and lateral electrostatic repulsion between enzyme molecules.

Interactions with OH-SAMs, are possibly mediated by hydrogen bonding, since under the pH range used the hydrogen of the OH- group (pKa=13) is retained.[35] In what concerns adsorption to COOH-SAMs, as the pH decreases from 8 to 4.5 the COOH- groups become less ionised and the overall charge of the surface less negative, but GCR becomes more positively charged. Electrostatic interactions are thus probably favoured at pH 6.0 and 5.5, where the enzyme and the surface present opposite charges. Since the ionisation of COOH- at pH 4.5 is negligible,[35] interaction between GCR and COOH- at that pH may be mediated by hydrogen bonding. Nevertheless, the adsorption amounts obtained for these two types of SAMs at all pH used are similar, probably because electrostatic interactions provide relatively low contributions to protein adsorption at ionic strength >100 mM due to the

screening of protein charge by counterions.

The specific activity of GCR adsorbed on gold and on OH- and COOH-SAMs at pH 7-8, near the enzyme IP, was always higher than that of the free enzyme. Its has been previously suggested[3] that pH affects the level to which native-state proteins undergo structural alterations upon adsorption to a solid surface, and that structural rearrangements are minimal when adsorption occurs at a pH equal to the IP. The enzyme activation may have several explanations. Possibly, the interface brings the enzyme in contact with the substrate at a higher concentration and/or in a more favourable orientation/conformation for efficient catalysis. Several studies showed that purified GCR exhibits a very low level of activity *in vitro* and that a micellar or liposomal interface is needed for reconstitution of its catalytic function.[23,36] Presumably, anionic detergents like taurocholate, and also some acidic phospholipids, facilitate the interaction of the enzyme with the substrate by inducing a conformational change that conforms it into a more active state.[23] The activation of many lipases by an interface, the so-called interfacial activation, has also been extensively reported.[9,10] A similar mechanism could eventually explain the enhancement of GCR activity in the adsorbed state.

CONCLUSIONS

The adsorption of the enzyme glucocerebrosidase (GCR) to self-assembled monolayers (SAMs) of different functionalities (X= CH_3-, OH- and COOH-) was evaluated as a function of solution pH. Maximal GCR adsorption was observed on gold and on hydrophobic CH_3-SAMs. The extent of adsorption on this type of surfaces was strongly influenced by the solution pH. Lowering the pH from physiological to acidic values seems to trigger an increased solvent exposure of non-polar sites in the enzyme and modulate its adsorption behaviour. The specific activity of GCR adsorbed on gold and on OH- and COOH-SAMs was strongly dependent on the pH used. The activity was enhanced, compared to that of free enzyme, when adsorption was performed at a pH near the enzyme isoelectric point.

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Chapter IX

Preliminary studies on the adsorption of glucocerebrosidase to calcium phosphate microspheres

ABSTRACT

In this study the ability of hydroxyapatite (HAp) and calcium titanium phosphate (CTP) microspheres to reversibly adsorb the enzyme glucocerebrosidase (GCR) while preserving its biological activity, and to efficiently deliver it into GCR-deficient fibroblasts from GD patients was analysed. The amount of adsorbed GCR was determined by quantifying GCR depletion from the supernatants using the bicinchoninic acid assay. GCR release was monitored in cell culture medium by radiolabelling. Internalisation of GCR by GD fibroblasts was estimated by measuring the intracellular enzymatic activity before and after treatment with GCR-loaded microspheres. Under the conditions tested, CTP microspheres adsorbed ca. 3.6-fold more GCR than HAp microspheres. The activity of the adsorbed enzyme was higher than the control in the case of CTP microspheres and lower when HAp was used. GCR release from both types of microspheres was characterised by the elution of a large percentage of enzyme within 1 h of incubation, followed by a delayed release that extended for at least 30 days. Released GCR was internalised by GD fibroblasts increasing the intracellular enzymatic activity. In cells treated with the same amount of GCR-loaded CTP microspheres or free-GCR a higher intracellular activity was detected in the former case, suggesting that enzyme-loaded microspheres are more effective than the free enzyme.

Keywords: Enzyme adsorption, microspheres, hydroxyapatite, calcium titanium phosphate

INTRODUCTION

Successful reconstruction of bone defects remains a major concern in orthopaedic surgery. Micro or nanoparticles suspended in autologous blood or other appropriate vehicle have been used as injectable bone fillers.[1, 2] Upon implantation, the microspheres-vehicle system is expected to easily conform to the irregular implant site, providing a permissible matrix for both tissue and vascular ingrowth, as required for effective healing. In addition, depending on the specificity of the illness, microparticles can be loaded with bioactive agents to accelerate the process of tissue regeneration. The preparation and characterisation of hydroxyapatite (HAp) and calcium titanium phosphate (CTP) microspheres with a uniform size have been previously described,[3] and *in vitro* studies revealed that they can be used as supports for the culture of osteoblastic-like cells.[1,2]

In this study, HAp and CTP microspheres were analysed as vehicles for the recombinant enzyme glucocerebrosidase (GCR). This enzyme is used in the treatment of Gaucher disease (GD, type I), which is characterised by a number of severe disabling symptoms, including bone pathologies.[4] Enzyme-loaded microspheres could be used to fill GD bone defects and contribute to their treatment, by providing a physical support for new tissue formation and localised enzyme delivery.

Most of the techniques used in the preparation of protein delivery systems rely on the association of proteins with soluble or solid carriers. There are several protein-carrier coupling strategies, such as: entrapment in matrix or reservoir systems, covalent binding to a surface and physical adsorption. These methods have been applied particularly within the field of enzyme immobilisation[5] and, more recently, for controlled release of protein pharmaceuticals.[6]

Non-specific physical adsorption of a protein from a solution on a solid surface is probably the simplest and longest known method of immobilisation. The ability to load the protein in a simple and cost-effective process, without using solvents or polymerization, is one of the greatest advantages of this method. Physical adsorption methods have been widely used in the development of microparticulate carriers for the localised delivery of vaccines to the site of action,[7-10] and for the delivery of proteinaceous agents in tissue regeneration applications.[11-14]

The objective of the present study was to investigate whether simple adsorption of GCR to

the surface of calcium phosphate microspheres could be used to immobilise the enzyme in a reversible way while preserving its biological activity, and allow it to be efficiently delivered it to GCR-deficient fibroblasts from GD type I patients.

MATERIALS AND METHODS

Preparation and characterisation of calcium phosphate microspheres

Calcium titanium phosphate [CTP, $CaTi_4(PO_4)_6$] and hydroxyapatite (HAp) microspheres were prepared as previously reported.[3] The ceramic powders were mixed at a ratio of 0.2 (HAp) or 0.4 (CTP) with 2% w/v sodium alginate solution. After homogenisation, the paste was extruded drop-wise into a 0.1 M $CaCl_2$ crosslinking solution, where spherical-shaped particles instantaneously formed and were allowed to harden for 30 min. The size of the microspheres was controlled by regulating the extrusion flow rate using a syringe pump and by applying a coaxial air stream (Encapsulation Unit Var J1; Nisco). At completion of the gelling period, microspheres were recovered and rinsed in water in order to remove the excess $CaCl_2$. Finally, they were dried overnight in a vacuum-oven at 30ºC, and then sintered at 1100ºC for 1h, with a uniform heating rate of 5ºC/min from room temperature. The size of the microspheres was estimated using an inverted platen microscope (Olympus PME3-ADL) equipped with an ocular micrometer with an accuracy of 10 μm. Specific surface areas were measured by the Brunauer, Emmel and Teller (BET) method. Zeta potentials (ZP) of CTP and HAp microspheres were measured at pH 7.0 in 10^{-4} M KCl with a EKA ElectroKinetic Analyser (Anton Paar).

Preparation of GCR solutions

Enzyme stock solutions (2 mg/mL) were prepared by reconstituting recombinant human glucocerebrosidase (GCR, Genzyme Corporation) lyophilised powder in distilled-deionised water. The stability of CGR in the stock solution is maintained for more than one month at 4ºC. The final concentration of the GCR solution was confirmed using the bicinchoninic acid (BCA) assay.[15] The stock solution was diluted to 0.1 mg/mL immediately prior to use, in phosphate-buffered saline (PBS, pH=7.4).

Adsorption of GCR to microspheres and enzymatic activity assay

Microspheres were incubated with GCR solutions under rotation and end-over-end mixing for 30 min at room temperature. At the end, the supernatants were withdrawn and the microspheres were washed 3 times with PBS to remove unbound GCR. The concentration of GCR solutions before and after adsorption was determined by the BCA assay. The catalytic activities of the adsorbed and free enzyme were determined using a method previously described.[16] Briefly, the enzyme was incubated with 5 mM 4-MU-Glc in 50-100 mM citrate-phosphate buffer (pH 5.5), for 30 min in a humidified atmosphere at 37ºC. The reaction was stopped with 1 M glycine in 30% w/v NaOH (pH 10.3). The product 4-methylumbelliferone (4-MU) was quantified by fluorescence spectroscopy with excitation at 366 nm and emission at 445 nm, using commercial 4-MU (Sigma) as a standard. The Michaelis-Menton constant (K_m) was determined from a Lineweaver-Burk plot using varying concentrations of the substrate (1.56-25 mM).

Release of GCR from calcium phosphate microspheres

This study was performed using ^{125}I-GCR, which was radiolabelled using the Iodogen method as previously described.[17] ^{125}I-GCR loaded calcium phosphate microspheres were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v foetal bovine serum, 1.5% v/v Ultraser G, 2.5 μg/mL fungizone, 200 U-200 μg/mL peniciline-streptomicine and 50 μg/mL kanamicine (standard culture medium) at 37ºC in a humidified atmosphere of 5% v/v CO_2 in air. At different time points, the supernatants were recovered, centrifuged (5 min at 5,000 rpm) to remove ceramic particles and counted for radioactivity in a γ-counter. Fresh medium was added. This assay was prolonged for 30 days.

Cell cultures

Human fibroblasts from patients affected by type I GD (GCR-deficient cells) were obtained from skin explants under consent of the local Ethics Committee, and cultured as described elsewhere.[18] Cells were routinely maintained at 37ºC in a humidified atmosphere

of 5% v/v CO_2 in air in standard culture medium. The medium was renewed every 3 days. For subculture, cells were rinsed twice with warm PBS and harvested with 0.25% w/v trypsin-0.05% w/v ethylenediaminetetraacetic acid (EDTA). Cells were centrifuged (7 min, 1,100 rpm), resuspended in fresh standard culture medium and used immediately.

Uptake of GCR by GD fibroblasts

Cells were seeded at 3×10^4 cells/cm^2 in 24-well plates and incubated for 24 h with different amounts of free-GCR (from 1 to 30 μg) or GCR adsorbed to calcium phosphate microspheres. Untreated cells from type I GD patients were used as a reference. Microspheres without adsorbed GCR and GCR-loaded microspheres in the absence of cells were used as controls. At the end of the incubation period, the supernatants were discarded and cells were rinsed twice with PBS and brief sonicated in 0.1% v/v Triton X-100. The homogenates were centrifuged (5 min at 10,000 rpm and 4ºC) to remove ceramic particles and cell debris. The supernatants were used for GCR activity measurements as described above but in the presence of 6 mg/mL of sodium taurocholate.

RESULTS AND DISCUSSON

Adsorption of GCR to calcium phosphate microspheres

Microspheres with a uniform size of 600±23 μm (CTP) and 535±25 μm (HAp) were obtained. Both types of microspheres presented an interconnected microporosity and their specific surface areas were 0.27 m^2/g for the CTP microspheres and 0.68 m^2/g for the HAp microspheres, as determined by the BET method. Further details on the physicochemical and morphological characterisation of the microspheres are provided elsewhere.[3]

Adsorption of GCR was performed at pH 7.4, in the vicinity of the enzyme isoelectric point IP, which occurs around 7-8, where it exhibits higher structure stability and therefore a smaller tendency to denaturate upon adsorption.[19] Although GCR adsorbed to both types of microspheres, the properties of the materials largely influenced the amount of adsorbed enzyme. Under the conditions used, CTP microspheres adsorbed 36 ± 5.0 ng GCR/cm^2,

while HAp microspheres adsorbed 10 ± 2.0 ng GCR/cm^2. These figures refer the real surface are of the microspheres. The differences in the adsorbed amounts may be related to differences in protein-surface electrostatic interactions. Zeta potential (ZP) measurements showed that at physiological pH both types of microspheres are negatively charged, the ZP of CTP microspheres (-54.33 ± 6.35 mV) being more negative than the one of HAp microspheres (-15.96 ± 2.12 mV). Although at physiological pH the enzyme exhibits a neutral net charge, it has positively and negatively charged residues that may mediate adsorption. In this study only an enzyme concentration and an adsorption time were used and the choice of these parameters was based on a previous work.[17]

The catalytic activity of adsorbed GCR was strongly depended on the type of ceramic used. Results are expressed as a percentage of the activity obtained with the same concentration of free-GCR. For GCR adsorbed on CTP microspheres the activity was higher than that of the control (367.1 ± 87.3%), while in the case of HAp microspheres it was lower (9.5 ± 3.0%). Similar results were previously reported concerning the adsorption of GCR to CTP and HAp powders.[20] Activation of GCR by CTP microspheres was further confirmed by calculation of the Michaelis-Menton constant (K_m). A K_m= 9.0 mM was obtained for the adsorbed enzyme while a K_m= 30.5 mM was obtained for the free enzyme. This parameter is roughly an inverse measure of the affinity or strength of binding between the enzyme and its substrate, and so the lower the K_m, the greater the affinity.

Even if the integrity of the enzyme is preserved, a certain degree of inactivation is usually expected in immobilised enzymes. It may result, for example, from a decreased substrate-accessibility due to diffusional limitations or from an inadequate orientation of the enzyme active site at the surface.

The enhancement of enzymatic activity, such as that reported above, is a less common effect, although the activation of many lipases by an interface, the so-called interfacial activation, has been reported.[21,22] It is well documented that purified GCR exhibits a very low level of activity *in vitro* and that a micellar or liposomal interface, is needed for reconstitution of its catalytic function.[23] Presumably, this interfaces facilitates the interaction of GCR with the substrate by inducing a conformational change in the enzyme that converts it into a more active state.[23] A similar mechanism could eventually explain the enhancement of GCR activity in the adsorbed state, as already suggested in a previous work.[17] Activation of GCR by liposomal vesicles is only observed when phospholipid with

negatively charged polar groups are used.[23] Under the acidic conditions of the activity assay (pH 5.5), the surface of the CTP microspheres (IP around 3)[24] is negatively charged while the HAp microspheres (IP around 6)[24] is slightly positive, which may partially explain the results obtained.

Release of GCR from calcium phosphate microspheres

GCR release from calcium phosphate microspheres was monitored in cell culture medium supplemented with serum, to simulate a physiological environment and also to reproduce the conditions used in the subsequent cell culture studies. Upon implantation, microspheres will be in contact with plasma containing proteins and different ions, which are likely to influence GCR displacement from the surface of the vehicle.

Desorption of proteins from solid surfaces may be driven by different factors.[25-28] For example, it may be promoted by replacing the bulk solution used during adsorption by another of different ionic composition, pH and/or increased ionic strength.[28] Adsorbed proteins may also be exchanged to varying degrees by other proteins present in solution if they have a stronger affinity for the surface.[25-28] Results are presented in Fig. 1. Upon incubation, a large percentage of the enzyme was rapidly eluted from the microspheres within 1 h. After this initial stage, there was a delayed release of GCR, which extended for at least 30 days. At this point, the amount of released GCR in relation to that initially present in the microspheres was ca. 50% for both types of microspheres. In order to attenuate the initial burst effects and obtain a more sustained release, some authors have coated ceramic microspheres with polymers, such as polylactic acid or polyethylene vinyl alcohol.[11,12]

The occurrence of two distinct kinetic stages during GCR desorption from the microspheres may have several explanations. A fraction of the enzyme may be present inside the pores of the microspheres (absorbed) and not adsorbed to the surface, being rapidly eluted.

Moreover, the adsorbed enzyme molecules may present different conformational states.[28] Upon incubation in a solution containing a desorbing agent the enzyme molecules that are adsorbed by weaker forces may be easily displaced from the surface and transferred to the bulk solution by diffusion. Desorption of loosely attached proteins leaves a fraction of the

interface available for the lateral expansion of the remaining adsorbed proteins.[28] As a result, these protein molecules may alter their conformation and establish more contact points with the surface. These strongly bound molecules are expected to desorb at a slow rate or even become irreversibly adsorbed.[28]

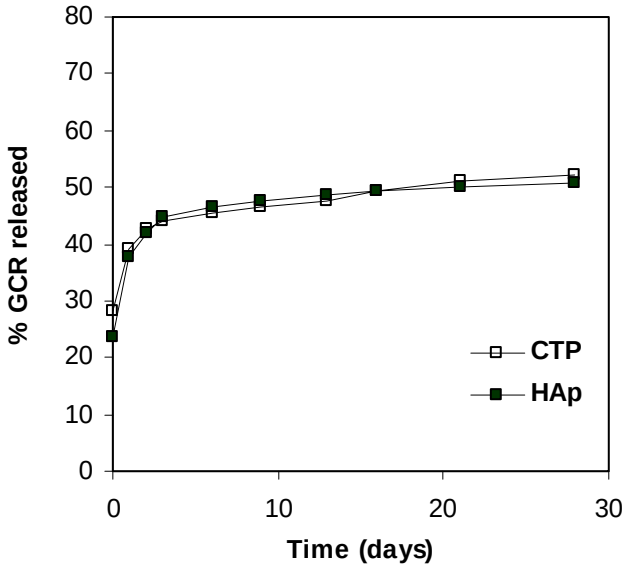

Figure 1. Percentage of GCR released from CTP and HAp microspheres into cell culture medium supplemented with serum during 30 days of incubation.

Uptake of GCR by GD fibroblasts

For their application as a delivery system, the microspheres must be able to bind GCR and release it in a biologically active conformation.In order to evaluate the integrity of the released enzyme, GD fibroblasts were incubated with GCR-loaded microspheres and after 24 h the increase on intracellular enzyme activity (internalised enzyme) was measured. Since the different materials result in different quantities of adsorbed GCR per mg of microspheres, cells treated with different quantities of free-GCR were used as control. As shown in Fig. 2, by increasing the amount of free-GCR in the medium from 1 μg to 30 μg, the level of GCR intracellular activity in cells increases in a dose-dependent manner, without reaching saturation.The values of intracellular GCR activity (IA) were related with the amount of free-GCR by mathematical fitting (Fig. 3).

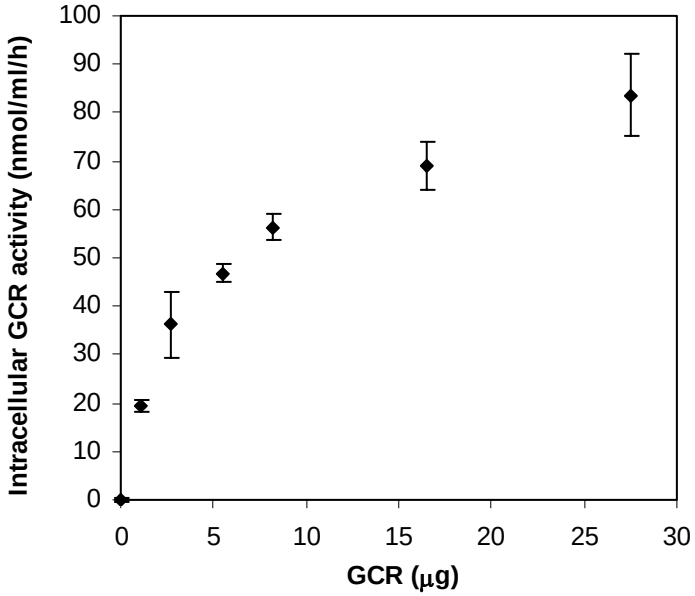

Figure 2. Uptake of free-GCR by GD fibroblasts as a function of enzyme amount in the medium. Cells were incubated at 37ºC for 24 h with 1-30 μg of free-GCR. Results are presented as mean ± standard deviation (n=3).

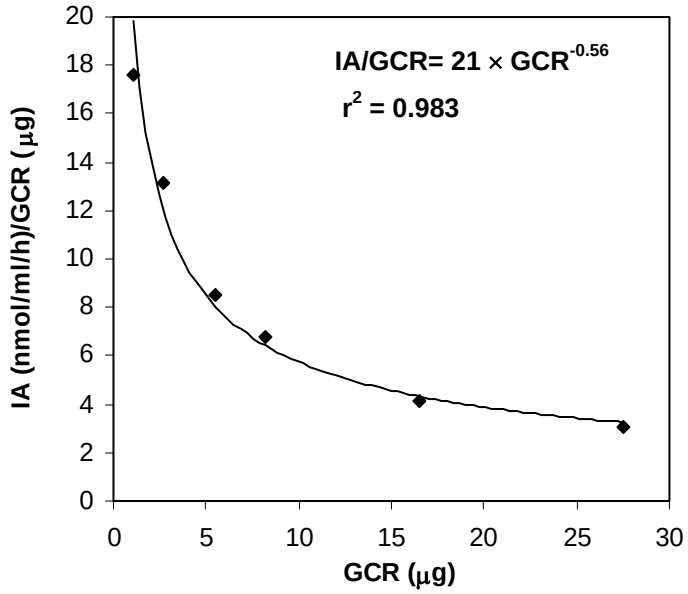

Figure 3. Relationship between the intracellular GCR activity (IA) and the amount of free-GCR in the medium: Experimental data (♦) and mathematical fitting (—).

The equation: $IA/GCR = 21 \times GCR^{-0.56}$ was then used to generate the values of intracellular activity that would be obtained if cells had been treated with amounts of free-GCR matching the amounts of adsorbed GCR (total loaded enzyme) for the two materials. In this way the effects of treating cells with adsorbed-GCR or exactly the same amount of free-GCR (control) can be compared. Results are presented as a percentage of the control (Fig. 4). When GCR-loaded CTP microspheres were used, the increase in intracellular GCR activity was 170 ± 3 % of the control. In the case of HAp microspheres the increase of intracellular activity represented only 20 ± 13 % of the control.

These values were, in principle, underestimated, since a fraction of the total loaded enzyme was probably lost during washing, while another fraction (ca. 50%, as suggested by the previous release studies) remained adsorbed to the microspheres and thus unavailable for uptake.

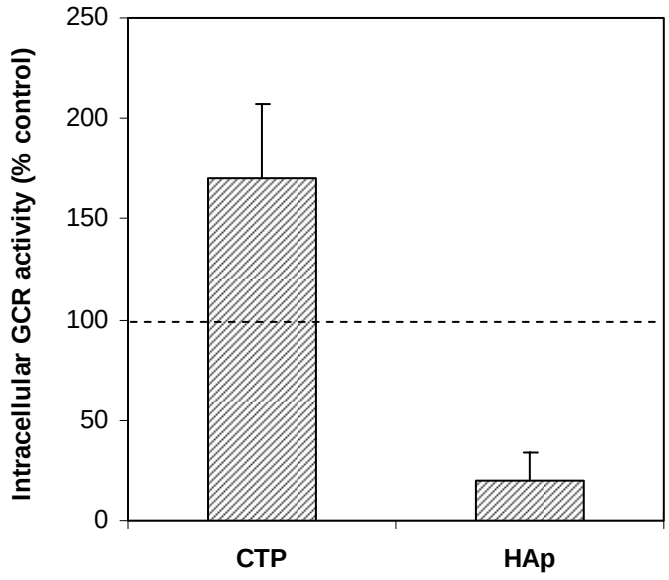

Figure 4. Intracellular GCR activity in cells treated with GCR-loaded CTP or HAp microspheres, depicted as a percentage of the control (cells treated with the same total amount of free-GCR). Results are presented as mean ± standard deviation (n=2).

Taken together these findings suggest not only that GCR maintains its integrity upon adsorption, but also that enzyme-loaded microspheres are more effective than the free enzyme, at least in the case of CTP microspheres.

The reasons for this still remain unclear. However, we have verified that upon incubation

at the conditions used (37ºC in complete cell culture medium) free-GCR becomes rapidly inactivated, exhibiting a very significant decrease in biological activity over time, with a half-life of ca. 60 min (data not shown). The low stability of purified GCR in solution, especially at pH ≥7.4, and even in the presence of stabilisers was also reported by Xu *et al*.[29] Moreover, other authors reported that the enzyme is susceptible to oxidation and self-aggregation.[30] So, it is reasonable to assume that the vehicle provides a more favourable microenvironment to the enzyme, protecting it from the deleterious external conditions, for example by preventing structural rearrangements and denaturation. It appears that association with the carrier potentiates the biological effect of the enzyme under physiological conditions.

CONCLUSIONS

Physical adsorption provides a simple way to couple the enzyme GCR to HAp and CTP microspheres. The properties of the materials largely influenced the amount and activity of the adsorbed enzyme. CTP microspheres adsorbed higher GCR amounts for unit surface area than HAp microspheres. The activity of the adsorbed enzyme was enhanced in the case of CTP microspheres and was partially lost when HAp was used. When incubated in cell culture medium, the enzyme is released for at least 30 days from both types of microspheres. Cell culture studies using GD fibroblasts showed that GCR-loaded CTP microspheres are more effective than the free enzyme, rendering these microspheres potentially useful for *in vivo* localised enzyme delivery.

The adsorption-approach, which is attractive mainly due to its simplicity and reproducibility, has proven, in this particular study, to be effective in improving the activity of the enzyme.

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Chapter X

Concluding remarks and perspectives

1. Concluding remarks

The main objective of this thesis was to develop microparticles, with a spherical shape and a narrow size distribution, to be used as injectable fillers for the regeneration of bone defects and localised delivery of enzymes. As a specific application, the use of the system as an adjuvant therapeutic strategy to treat bone lesions associated with Gaucher disease (GD) was investigated, and microspheres were analysed as vehicles for the recombinant enzyme glucocerebrosidase (GCR), currently employed in its treatment.

Bone lesions are among the most debilitating manifestations of GD, and have an enormous impact on patient's quality of life by restricting mobility and interfering with the day by day activities.[1, 2] Skeleton response to enzyme replacement therapy (ERT) is slow and incomplete, particularly in the case of adults with lesions in an advanced state of progression.[3] This is indicative that new adjuvant therapies must be developed to restore bone function more rapidly and/or to a greater extent than with the currently available ones.

In association with ERT, the system proposed in this work could potentially contribute to repair GD bone-defects, namely those more susceptible to fractures, in two different ways:

- By acting as a bone-filler to provide a physical support for new tissue formation, guide the process of tissue development, and even elicit specific cell functions;
- By providing localised delivery of glucocerebrosidase to bone, reducing its overall systemic exposure. The use of a vehicle may, in addition, enhance the therapeutic potential of the enzyme by functioning as a protective matrix, since it is known that purified GCR is highly unstable in solution under physiological conditions, and is susceptible to oxidation and aggregation.[4, 5]

The use of an injectable material, which gives the possibility of filling bone-defects through a minimally invasive surgery, is particularly relevant when applying it to the treatment of GD bone lesions. GD patients suffer from haematological abnormalities, namely from disorders of the coagulation system that often result in a tendency for excessive bleeding,[6] which could pose serious problems if the material had to be implanted by a traditional surgical procedure.

To achieve these goals, the developed microspheres had to fulfil the following requirements: be small enough to be injected; have the ability to reversibly immobilise the enzyme GCR while preserving its biological activity; and be able to support osteoblastic-like cells adhesion, proliferation and differentiation.

During this research work, different types of microspheres were sequentially prepared: polymeric, polymer-ceramic and ceramic based. In all the cases, the ability of alginate to instantaneously form a hydrogel matrix in the presence of divalent ions was exploited, and microspheres were obtained by droplet extrusion of an alginate-containing paste under a coaxial air flow, followed by cross-linking of the polymer with Ca^{2+}. Besides its simplicity, some advantages of the selected technique include the possibility of carrying out the process at room temperature and in the absence of organic solvents, which makes it suitable for enzyme entrapment purposes, and of allowing microspheres to be recovered without the need for fastidious washing processes. To obtain ceramic microspheres, this process was followed by a sintering stage, to burn-off the polymer and bond together the ceramic granules, while maintaining the spherical shape of the particles. The described methodologies enabled the preparation of spherical particles with a uniform size, from c.a 350 μm to up 2000 μm, which may present some advantages compared to other types of microparticles described in the literature.

For glucocerebrosidase immobilisation, two different coupling techniques were selected, depending on the type of microspheres. GCR entrapment was used in the case of alginate and calcium phosphate-alginate microspheres, and GCR adsorption was used in the case of calcium phosphate powders and microspheres. By selecting the appropriate conditions, the different matrices and immobilisation strategies used allowed GCR to be loaded with none

or minor losses of integrity, and gave the possibility of obtaining different release kinetics. Whether or not a specific release profile is the more adequate for the therapeutic efficiency of GCR in the *in vivo* targeting of bone resident Gaucher cells needs to be evaluated. However, the possibility of modulating the release kinetics may allow the optimization of the system and expand the range of applications.

An important finding was that, upon adsorption to a solid surface, not only GCR integrity could be preserved, but in certain cases its catalytic efficiency could even be improved. The mechanisms that underlie this enzyme activation are not clearly understood yet. However, it is well established that purified GCR exhibits a very low level of activity *in vitro* and that a micellar or liposomal interface is needed for reconstitution of its catalytic function.[7] Presumably, anionic detergents like taurocholate, and also some negatively charged phospholipids, facilitate the interaction of the enzyme with the substrate by inducing a conformational change that converts it into a more active state.[7] The activation of many lipases by an interface, the so-called interfacial activation, has also been extensively reported.[8] A similar mechanism could eventually explain the enhancement of GCR activity in the adsorbed state.

From the manufacturing point of view, the adsorption immobilisation approach offers a number of important advantages, it is simple, rapid and highly reproducible, which makes it very attractive for biomedical applications.

The *in vitro* biological performance of the system was assessed using GCR-deficient fibroblasts obtained from a GD patient. Although not being the relevant target cells, fibroblasts were used because they are routinely cultured for diagnostic purposes and thus are readily available. Obtaining sufficient amounts of bone Gaucher cells, which would be ideal or, in alternative, of Gaucher macrophages from other tissues or derived from peripheral blood monocytes would be extremely difficult, if not impossible. However, since fibroblasts also internalise recombinant GCR, through yet unknown mechanisms, they were considered to be an adequate model for the studies conducted to evaluate the integrity of GCR after being released from the different matrices. The objective was not to establish the correct GCR dosage, which is largely an *in vivo* problem and was out of the scope of this study.

The behaviour of bone cells cultured on the different types of microspheres was also investigated. Because the ability of cells to attach, adhere and spread will influence their capacity to proliferate and differentiate in contact with the material, this initial phase is of critical importance.[9] Preliminary adhesion studies were performed using the MG63 human cell line, which expresses a number of features characteristic of relatively immature osteoblasts, and has been considered as an adequate cell culture model for this type of studies.[10] However, results showed that cell response to alginate-containing microspheres was not satisfactory. Cells were unable to interact with alginate microspheres, probably due the high hydrophilic nature of the polymer,[11] and were only able to spread and adopt a flattened morphology on calcium phosphate-alginate microspheres with a high percentage of ceramic.

In what concerns cells response to calcium phosphate microspheres, some preliminary studies with HAp microspheres were also carried out using MG63 osteoblastic cells. These studies were important to establish the ideal methodology for culturing cells on the surface of microspheres, namely in what concerned manipulating the microspheres, and obtaining a uniform and sufficient density of attached cells.

A more complete investigation of cell behaviour was only carried out with CTP microspheres, using primary cultures of bone marrow cells, since HAp is a very commonly used ceramic that is already well studied. Bone marrow has long been recognized as a source of progenitor stromal cells that can be induced to differentiate along the osteoblastic lineage under appropriate conditions. When cultured on CTP microspheres, cells were able to attach, adopt a typical osteoblast-like morphology and gradually proliferate. The surface of the microspheres became almost completely covered by dense layers of bone cells and a fibrillar extracellular matrix. Cells were able to bridge adjacent microspheres forming cell-microspheres clusters, and expressed bone phenotypic markers, which confirmed that differentiation along the osteoblastic lineage occurred.

A synergistic effect between the chemical composition of the microspheres and their structural features, namely the surface roughness, may possibly underlie these promising results. On one hand, several reports on the positive effects of different types of calcium phosphates, and in particular of CTP, on osteogenic differentiation may be found in the literature.[12, 13] On the other hand, it is well known that surface roughness modulates the expression of phenotypic markers, and enhanced differentiation of osteoblast-like cells on

rough surfaces in comparison with smother ones has been already reported by several authors.[14]

In view of these findings, from the different types of microspheres developed during this investigation, calcium phosphate microspheres appeared to be the most promising for the envisaged application as they fulfilled all the requirements already state. The best results in terms of GCR immobilisation were obtained when calcium titanium phosphate was used as the ceramic phase.

2. Perspectives

The research conducted during this thesis has put in evidence some of the potentialities of the developed microspheres, which may find applicability as bone-defects fillers and enzyme delivery vehicles to be used in several orthopaedic surgical procedures. Nevertheless, several topics still need to be addressed and should be investigated in the future, namely:

- **Injectability of the microspheres:** in order to be implanted through a minimally invasive surgery, the microspheres must be suspended in a vehicle with adequate rheological properties, which has to be selected and tested. The microspheres-vehicle system must be easily manipulated during surgery and adaptable to the irregular wound site, where it must remain for an adequate period of time. Several polymeric gels, autologous blood, and fibrin glues are some examples of vehicles that have already been used for the implantation of particulate materials.

- ***In vivo* osteogenic potential of the microspheres:** The *in vitro* studies performed during this investigation suggested that calcium phosphate microspheres are appropriate for culturing osteoblastic-like cells. However, the *in vivo* performance of the microspheres upon implantation on critical size bone-defects created in a suitable animal model must be investigated.

- **Evaluation of enzyme release kinetics and tissue distribution *in vivo*:** *In vitro* release studies are very useful to determine the reproducibility of the release kinetics, to analyse the integrity of the protein after interacting with the material, and to study the effect of formulation parameters on the system performance in a systematic way. Nevertheless, they often do not mimic the *in vivo* situation. For this reason, *in vivo* studies should be performed in order to establish the correct doses and release kinetics for the therapeutic efficiency of GCR in targeting bone resident Gaucher cells. However, no naturally occurring animals are presently available, since the lineage of the only one described was not maintained.[15] A mouse model of Gaucher disease was obtained in 1992 by targeted disruption of glucocerebrosidase gene.[16] However, due to the very severe phenotype, death occurs within the first 24 h of life, hence this animal is not a useful model to study new therapeutical approaches. Conduritol B-epoxide (CBE), an irreversible glucocerebrosidase inhibitor as been used to mimic some GD in mice.[17] More recently, a chimeric mouse model of Gaucher disease that reflects the increased glycolipids storage in the reticuloendothelial system has been described.[18] In the future, if one of these models proves to be useful to study bone alterations associated with GD *in vivo* studies may be carried out.

- **Physicochemical characterisation of the microspheres:** A more complete physicochemical characterisation of the microspheres should be carried out in the future, namely in what concerns the following parameters:

  Porosity: The CTP and HAp microspheres developed during this work present a uniform and interconnected porous network with different pores sizes, depending on the formulation. This feature, however, was not completely explored in the present work and a more complete characterisation of the porosity should be the object of future studies. Moreover, the possibility of obtaining different porosities, namely by varying the granulometry of the starting ceramic powders and the sintering conditions should also be investigated.

  Degradation behaviour: the *in vivo* degradation behaviour of the microspheres is also an important parameter that should be investigated. It has been repported that

the degradation of alginates is slow and uncontrollable.[19] However, modulation can be achieved by using, for example, partially oxidized alginates with bimodal molecular weight distributions.[19] In this case, the rate of degradation was found to be controlled by both the oxidation and the ratio of high to low molecular weights.[19]

In what concerns the ceramics used, while the degradation behaviour of hydroxyapatite has been already studied,[20] little is known about the degradation behaviour of calcium titanium phosphate. This calcium phosphate is expected to degrade at a slow rate,[21] which seems to be advantageous for the present application. Bone lesions associated with GD are frequently end-stage lesions with a very low regeneration potential, and so the material will probably have to remain within the cavity for long periods of time. It should be noted, however, that the versatility of the technique used to prepare the microspheres would enable other calcium phosphate materials to be used in alternative. This way, the degradation behaviour could in principle be tailored to a specific situation, thus expanding the range of applications of the system.

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